Final Report

DEVELOPMENT OF TECHNICS AND REAGENTS FOR ASSESSMENT OF VIRAL ECOLOGY

Job 1366

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TABLE OF CONTENTS

TABLE OF CONTENTS (contd)

1.0 TECHNICAL HIGHLIGHTS

The feasibility of the Passive Immunological Agglutination (PIA) system was examined using a myxovirus, adenovirus, echovirus, herpesvirus, coxsackievirus and one Mycoplasma species. The sensitivity of the PIA technique was compared with conventional assay techniques for detecting the presence of infective organisms. With the exception of the mycoplasma, the PIA system was shown to be up to 100 times as sensitive as the conventional assay system with a response time of 1-2 hours versus 2-14 days for the conventional methods.

The specificity of the PIA system was shown to be excellent for the agents tested. The effectiveness of the PIA system was analyzed by testing two groups of clinical specimens. The first group contained nine clinical specimens that were contractually required plus two additional specimens. Six of the required nine specimens were found to contain influenza A2 (HK) virus by the PIA method, and three proved to be negative. The additional two specimens were found to contain the virus (by PIA). The second group contained four specimens that were contractually required plus 5 additional specimens. All of the required four specimens gave a positive PIA test for influenza. The additional 5 samples included one that gave a positive PIA test for influenza.

The PIA technique was found to be superior to the conventional method for detecting the agents partly because of the much shorter total response time. Reagent stability was good for the seven months it was under examination.

The production of high titered antibody and its subsequent attachment to polystyrene latex particles has increased the sensitivity of the PIA test for mycoplasma from 500 cfu/ml to 100 cfu/ml.

Different readout systems were examined during the course of the program. Three systems appeared most conducive to automating the readout:

(1) an electronic particle connector

(2) a light scattering system

(3) and a microscope video computer system.

1.1 TASK SUMMARY

System Properties	Current Status	Objectives
Sensitivity:		
Myxovirus	100 EID_{50}/ml*	10-100 EID_{50}/ml
Herpesvirus	1 PFU/ml*	1-10 PFU/ml
Adenovirus	3 $TCID_{50}$/ml*	1-10 $TCID_{50}$/ml
Coxsackievirus	1 ID_{50}/ml*	1-10 ID_{50}/ml
Echovirus	1 PFU/ml*	1-10 PFU/ml
Mycoplasma	100 CFU/ml*	10-100 CFU/ml
Response Time:	1 hour	1 hour
Sensitized Beads Storage Stability	7 months tested	1 year
Specificity	High for all organisms tested	High specificity for all organisms

* EID_{50} - Egg Infectious Dose, 50% Response

PFU - Plaque-Forming Unit or Pock-Forming Unit

$TCID_{50}$ - Tissue Culture Infectious Dose, 50% Response

CFU - Colony Forming Units

ID_{50} - Infectious Dose (mouse), 50% Response

2.0 INTRODUCTION

The summary report on Aerojet-General Job No. 1366 is provided covering the period of June 19, 1969 to July 31, 1970.

This program is directed towards the development of the techniques and reagents necessary to make both qualitative and quantitative determinations of the microbial ecology of a crew during extended space flight. The first phase of this task is the investigation of feasibility of an antigen/antibody system utilizing the PIA (Passive Immune Agglutination) technique.

Investigations in recent years have shown that latex particles can be successfully used in agglutination tests for detection of various antigens and antibodies. The latex fixation technique was developed by Singer and Plotz, [1] who applied it for the serological diagnosis of rheumatoid arthritis. Two approaches were used to study subsequent agglutination reactions. In the first, antigen attached to the latex particle is used to measure agglutination quantitatively in the presence of test sera or other fluids. The second approach incorporates antibody attached to the particles to test for the presence of antigen in samples of unknown composition.

In the second approach, two methods of protein uptake are postulated: (1) protein is absorbed as a bimolecular layer where the second layer contains about half the number of molecules of protein as the first layer; (2) absorbed protein forms a random immobile film with the protein molecules oriented parallel to the latex surface. Further absorption in the latter case occurs in the interstices between molecules already absorbed, with the orientation of the secondary molecules now perpendicular to the plane of the styrene.

Several modifications of the latex agglutination reaction have been studied on this contract but the basic reaction principle remains the same; i.e., interaction of antigen and antibody in a particulatized (via latex spheres) suspension. The particles function to amplify the molecular interaction of antigens and antibodies to the level where the immunospecific reaction can be more readily observed. In this sense, the latex particles are a valuable, though simple, tool for detecting and quantitating

small amounts of either antigen or antibody in aqueous systems.

The PIA system utilizes the polystyrene bead coated with antibody.

To prove the feasibility of this system as a means of carrying out the desired determinations, six agents were chosen as model systems for study. The agents chosen were: (1) a myxovirus, influenza A_2, Hong Kong variety; (2) an adenovirus, type A_4; (3) Herpesvirus hominis; (4) an echovirus, type 9; (5) a coxsackievirus, type A_2; and (6) Mycoplasma pneumoniae.

The test agents selected provide an effective survey of the respiratory and enteric viruses usually encountered, as well as a survey of the usual cultural recovery techniques employed.

The MEMS feasibility study has been divided into two main sections. The first section contains the results of the first 9 months technical effort. The second section contains the results of the last three months technical effort which was added on contractually as a continuation.

The first section contains the results of the immunological agent propagation efforts used to conduct the feasibility study of the PIA technique, specificity examinations, reagent shelf life studies, and examination of nine NASA-supplied clinical samples.

The second section examines the effect of improved antiserum preparations to influenza and mycoplasma. In addition, an examination was made of readout systems that could be used to automate the PIA test. At present, conductometric or light scattering appear to be the method of choice.

3.0 TECHNICAL DISCUSSION FIRST 9 MONTHS

3.1 SYSTEM SENSITIVITY

The ultimate sensitivity of the PIA detection system is dependent on a number of factors: (1) the reaction rate between antigens and specific antibody to form stable complexes, (2) the amount of active antibody on the sensitized beads, (3) the concentration of sensitized particles, (4) the method of readout and (5) the amount of cross-reactivity.

The sensitivity of the PIA system was studied with five viruses and one Mycoplasma. It was the purpose of this study to determine the feasibility of the PIA test for a selection of viruses that would represent a cross-section of viruses encountered during clinical testing. Our results confirmed the excellent sensitivity of the PIA method.

The PIA test procedure is detailed in Appendix B.

3.1.1. COXSACKIEVIRUS, TYPE A_2

The detection of this virus presented some unusual problems because of the need for propagation of the virus in the brains of suckling mice. The brains are harvested and homogenized in agamma horse serum-saline solution for use as antigen. The brains of normal suckling mice, processed identically, are used as the controls for the PIA sensitivity examinations. The reason for the high control values obtained with this virus reflects the method of preparation of the antiserum received from NASA. Coxsackievirus antiserum was produced by injections of infected suckling mouse carcasses into rabbits. Thus, in addition to the antibody against the virus that was produced, there wre also large quantities of antibody against other mouse tissue antigens. It may be antibody against mouse tissue that accounts for the high background level and standard deviation that is seen in the PIA sensitivity determinations with the coxsackievirus.

In spite of the high background level, the sensitivity of the PIA test with the coxsackievirus appears to be on the order of 10^0 - 10^1 ID_{50}/ml. Table 1 is a summary table of data obtained in approximately 40 experiments (detailed in Table 2) and represents the type of sensitivity one can expect with the materials currently available. Figure 1 is a graphic representation of the average signal and noise data obtained in this system, and shows the formula for a best fit straight line.

Table 1

AVERAGE S-N OF PIA DETERMINATIONS WITH THE COX-SACKIEVIRUS AS MEASURED BY MEAN CLUMP SIZE

Virus Concentration ID_{50}/ml*	Signal (S)	Noise (N)	S-N
10^4	7.19	4.05	3.14
10^3	7.01	4.35	2.66
10^2	7.56	4.54	3.02
10^1	6.42	4.79	1.63
10^0	5.58	4.11	1.47

Average Noise = 4.3

Standard Deviation = 0.79

*Infectious Dose

TABLE 2

PIA SENSITIVITY DETERMINATIONS
COXSACKIEVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Coxsackievirus Concentration ID_{50}/ml*	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^4	5.90	4.90	1.00
10^4	7.72	4.38	3.34
10^4	7.15	4.04	3.11
10^4	6.05	3.55	2.50
10^4	6.13	4.41	1.72
10^4	8.55	5.00	3.55
10^4	5.08	4.11	0.97
10^4	9.05	4.56	4.49
10^4	7.60	4.06	3.54
10^4	8.55	2.69	5.86
10^4	7.70	3.26	4.44
10^4	7.26	4.07	3.19
10^4	6.79	3.63	3.16
10^3	7.16	5.25	1.91
10^3	6.60	4.08	2.52
10^3	5.36	3.69	1.67
10^3	5.18	3.83	1.35
10^3	8.60	5.54	3.06
10^3	7.30	3.87	3.43
10^3	8.92	4.20	4.72
10^2	7.48	5.40	2.08
10^2	8.21	3.89	4.32
10^2	4.84	3.74	1.10
10^2	5.65	3.75	1.90
10^2	9.04	6.75	2.29
10^2	7.38	4.50	2.88
10^2	8.40	4.00	4.40
10^2	8.05	5.04	3.01
10^2	8.99	3.75	5.24
10^1	5.46	4.29	1.17
10^1	8.32	5.72	2.60
10^1	8.27	3.83	4.44
10^1	4.97	4.58	0.39
10^1	5.12	5.53	-0.41
10^0	**6.07**	**4.59**	**1.48**
10^0	5.40	3.42	1.98
10^0	6.45	4.49	1.96
10^0	4.40	3.96	0.44

*Infectious Dose

Average Signal of PIA Sensitivity Determinations with the Coxsackievirus
As Determined by Mean Clump Size

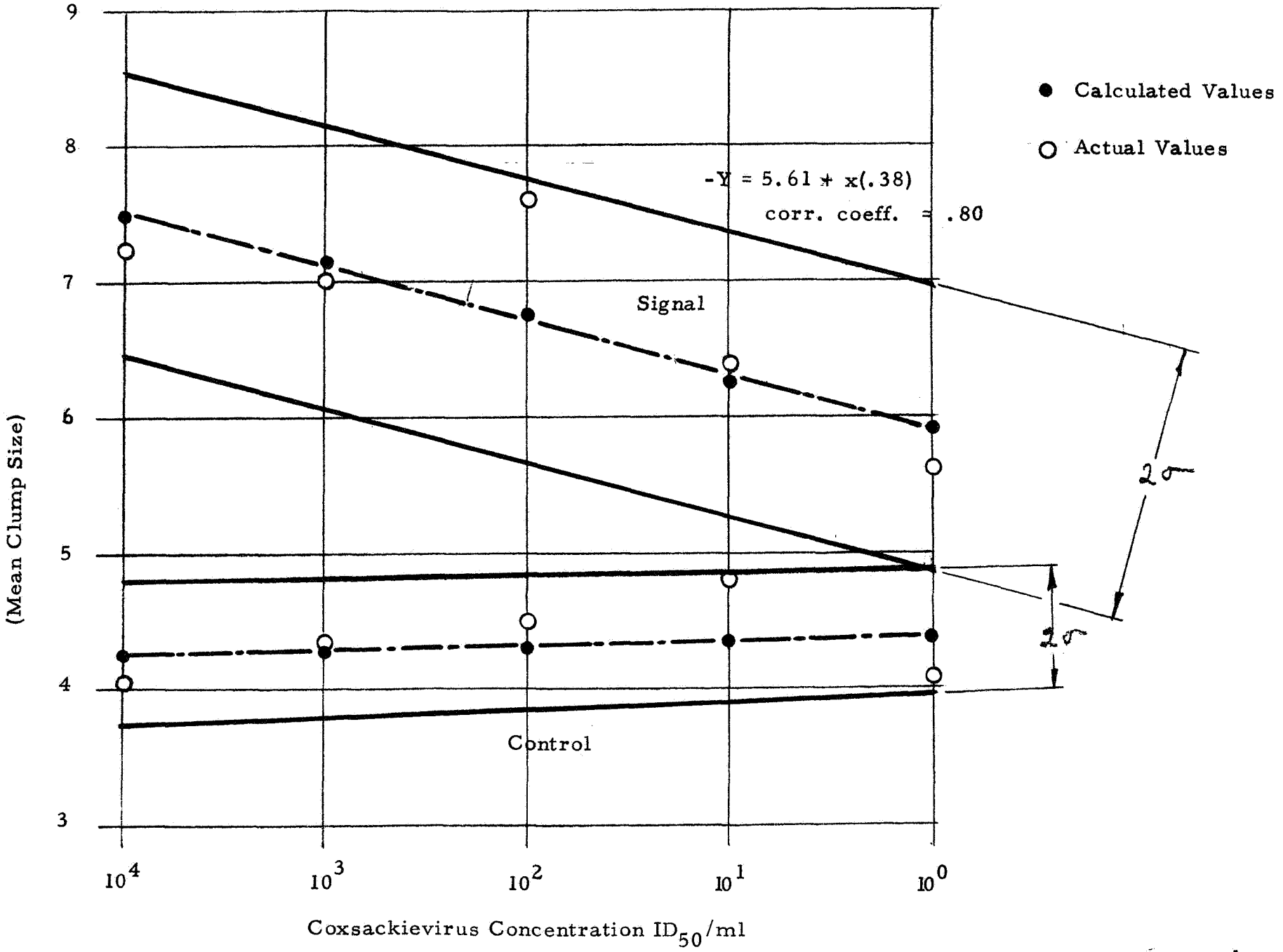


Figure 1

through the points with a one sigma (σ) bandwidth. The correlation coefficient of approximately 0.8 was obtained indicating that the line is a reasonable fit through these points. The sensitivity of the system is seen to be approximately 1 ID_{50} /ml.

The data seen in Figure 1 show little dose-response at concentrations of 10^4 - 10^2 ID_{50}/ml. These viral concentrations are apparently at the saturation level for this particular test. Under these conditions, it appears as if the maximum clumping occurs at a coxsackievirus concentration of 10^2 ID_{50}/ml. A partial explanation for this saturation or concentration effect is the lack of enough specific viral antibody on the surface of the particles to show a dose-response at higher antigen concentrations. As a result, the virus particles that are added at high concentration quickly attach to the specific sites on the beads, effectively saturating each bead combining site. This phenomenon acts to prevent the formation of bead/Ab/Ag/Ab/bead complexes.

From these data presented here, it is reasonable to expect that the sensitivity and reliability of this system can be improved. In our opinion, sensitivity determinations run with a single standardized preparation would increase the sensitivity of the PIA system for the coxsackievirus by a factor of 10. An additional ten-fold increase in sensitivity could be obtained with a sensitized bead preparation made with higher titer antibody.

3.1.1.1 PROPOGATION AND CHARACTERIZATION OF COXSACKIEVIRUS TYPE A2

The Sickle and Daldroff isolate of Coxsackievirus Type A2 was received in our laboratory from NCDC as a 20% suspension of the 7th passage in suckling albino mice. The seed had been assayed in suckling mice (10^7 ID_{50}/ml) on 7/21/67. Passage 1 in our laboratory was propagated from a 10^{-3} dilution of the seed. Aliquots of 0.01 ml each of the 10^{-3} dilution were injected intracerebrally (IC) into a sufficient number of 2 to 4-day old suckling albino mice to yield a harvest of 200 ml of 20% mouse brain suspension in agamma horse serum. The mice became moribund in 36 to 48 hours and were sacrificed after complete paralysis. The brains were removed and homogenized in a Waring blender. The tissue debris was removed by centrifugation at approximately 10,000 g for ten minutes.

Assay of the 20% mouse brain suspension was carried out in 2 to 4-day old suckling albino mice. Serial 10-fold dilutions were prepared in Hanks' Balanced Salt Solution (HBSS). Litters of not less than 5 mice each were innoculated IC in duplicate for each dilution with 0.01 ml of the proper dilution per mouse. The mice were observed twice daily for paralysis and ultimately death. Any deaths occurring in the first 24 hours postinoculation were considered as due to traumatic or nonspecific events. Mice innoculated with infectious coxsackievirus were moribund in 36 to 40 hours and dead in 48 to 64 hours postinoculation. The number of deaths in each dilution was recorded over a period of ten days. The titer was calculated by the Reed-Muench method as 4×10^6 ID_{50}/ml.

3.1.2 ECHOVIRUS, TYPE 9

The echovirus used in this study was propagated on African Green Monkey Kidney (AGMK) cells, and the antiserum used to sensitize the latex particles was obtained from (NASA (1:128 CF titer).

The detection sensitivity of the PIA system for the echovirus was evaluated in two parts, the first consisting of 26 tests and the second consisting of 31 tests. The reasons for this type of evaluation was to determine some of the non-biological factors affecting the background level and the sensitivity of the reaction. Each antigen/antibody system is slightly different not only because of the different sources of antibody for the different agents, but also because of the uniqueness of each viral antigen mosaic.

In the first series, 26 tests were performed with the echovirus. Figure 2 is a summary graph representing the averages of the first series tested. The experiments were conducted at four viral concentrations. This figure shows dose-response function. The formula for a best linear fit is given in Figure 2 and has a correlation coefficient of 0.81. Associated with these curves is a 1 sigma bandwidth. The data in Figure 2 show that the upper band of the control and the lower band of the signal intersect at an agent concentration of 3 PFU/ml.

The next series consisted of 31 tests. Figure 3 shows the signal curve and background curve, and the formula for a best-fit straight line through the signal curve for the second test series. The correlation coefficient of 0.88 represents a good fit through these points and shows an increased reliability over the first test series. Associated with these curves is a 1 sigma band around both the signal and control curves. In Figure 3, the upper band of the control curve and the lower band of the signal curve intersect on the abscissa at 1 PFU/ml. This compares to the 3 PFU/ml intersection obtained in Figure 2.

The curves in Figures 2 and 3 show some of the factors that are involved in sensitivity determinations that are not associated with the mechanics of the test itself. The increase in sensitivity obtained was due in large part to the decrease in fluctuation that occurred in the signal and control curves, probably because of the learning associated with repeated testing. This decrease in fluctuation was measured by the standard

Average Signal with the Echovirus as Determined by
Mean Clump Size
(1st Series)

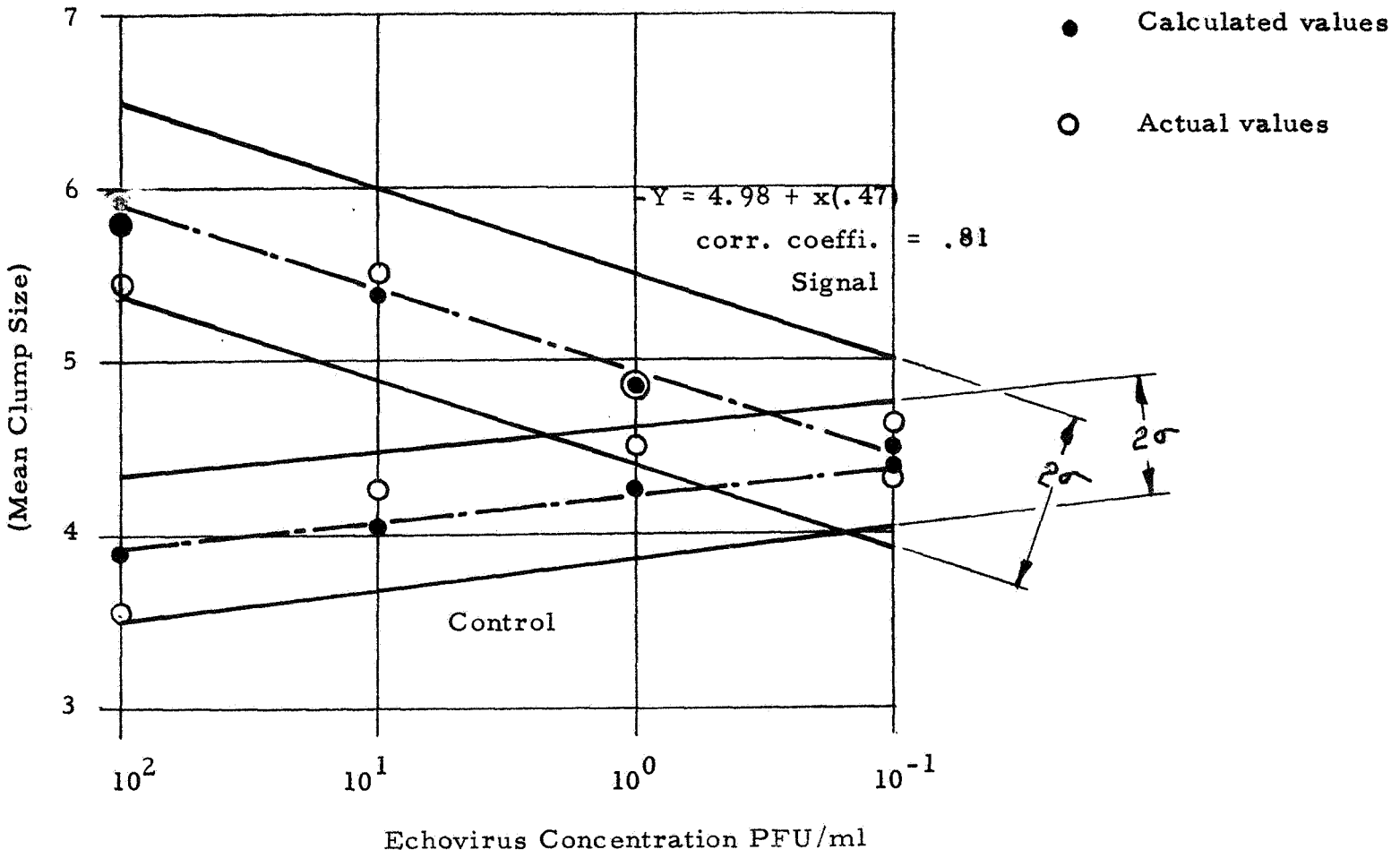


Figure 2

Average Signal with the Echovirus as Determined by Mean Clump Size)

(2nd Series)

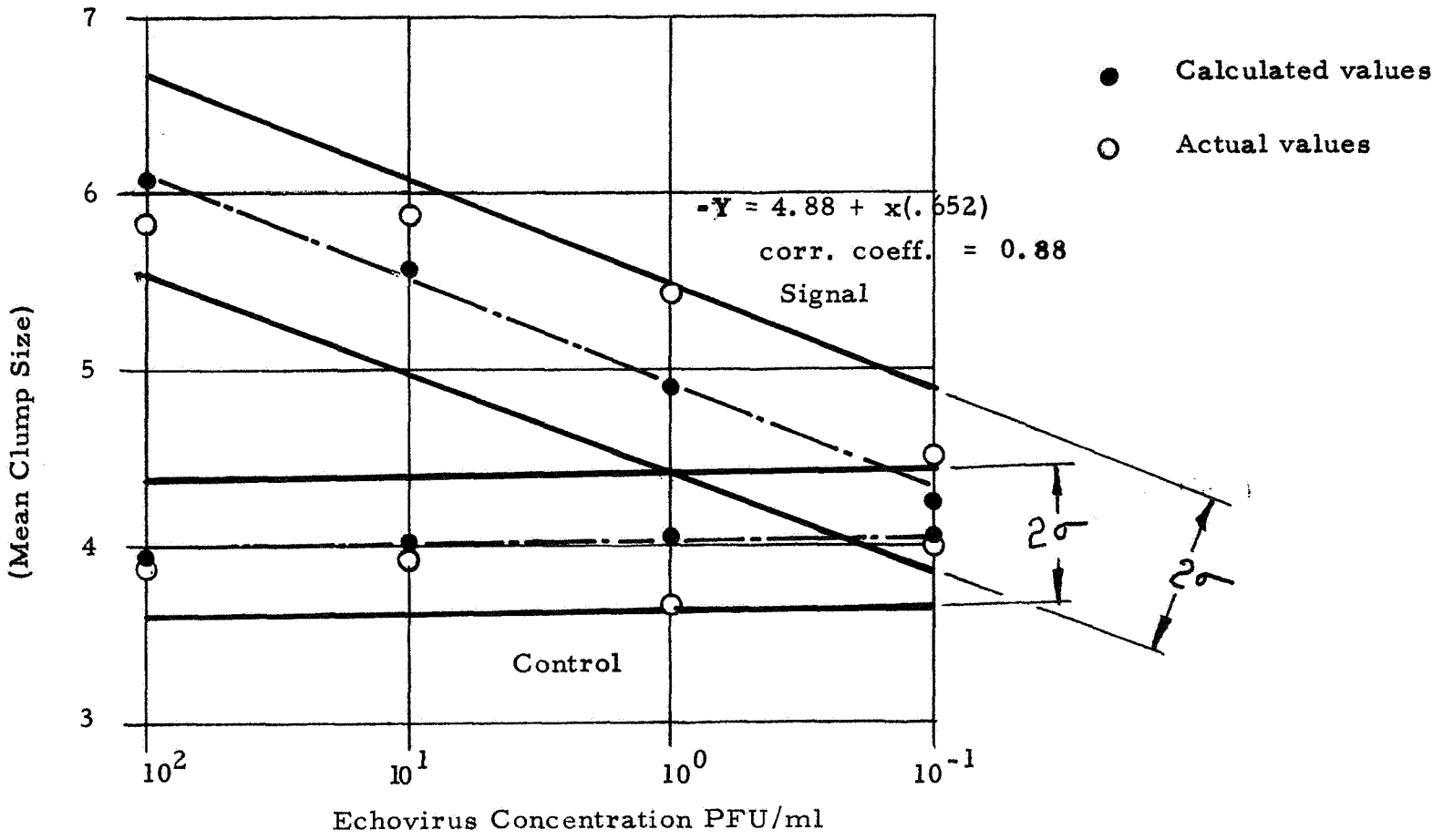


Figure 3

deviation associated with the average of the control points. In the second series of experiments, the absolute average control value was slightly reduced, but the fluctuation in the control values was reduced by approximately 33 percent. It was this reduction in fluctuation that accounted for the slightly increased sensitivity.

Tables 3 and 4 show the average results of the same two series of tests. Tables 5 and 6 show results of the individual experiments that were performed. In each case at a virus concentration of 10^3 PFU/ml, the signal is lower than at lower agent concentrations. This concentration effect has been noticed in other virus/antibody systems in our laboratory. Work with the mycoplasma has shown that increasing the titer of the antibody globulin can increase both the sensitivity and the concentration or dynamic range of the PIA system. The PIA test has been shown to detect the echovirus successfully, and it is felt that the concentration effect that is now observed at 10^3 PFU/ml can be alleviated with higher titered antibody. Likewise, the sensitivity of the system would be improved.

Table 3

Average S-N of PIA Determinations with the Echovirus as Measured by Mean Clump Size
(1st Series)

Echovirus Concentration, PFU/ml*	Signal (S)	Noise (N)	S-N
10^3	4.26	4.02	0.24
10^2	5.24	3.52	1.72
10^1	5.56	4.24	1.32
10^0	4.84	4.52	0.32
10^{-1}	4.55	4.33	0.22

Average Noise = 4.15

Standard Deviation = 0.61

*Plaque - forming units

Table 4

Average S-N of PIA Determinations with the Echovirus as Measured by Mean Clump Size (2nd Series)

Echovirus Concentration, PFU/ml*	Signal (S)	Noise (N)	S-N
10^3	3.58	4.11	-0.53
10^2	5.80	3.84	1.96
10^1	5.83	3.99	1.84
10^0	5.40	3.66	1.74
10^{-1}	4.21	4.52	-0.31

Average Noise = 3.96

Standard Deviation = 0.45

*Plaque - forming units

Table 5

PIA SENSITIVITY DETERMINATIONS WITH THE ECHOVIRUS AS DETERMINED BY MEAN CLUMP SIZE
(1st Series)

Echovirus Concentration PFU/ml*	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^3	4.98	4.21	0.77
10^3	4.38	3.51	0.87
10^3	4.83	4.06	0.77
10^3	2.82	3.89	-1.07
10^3	4.16	4.36	-0.20
10^3	5.05	3.69	1.36
10^3	3.63	4.40	-0.77
10^2	4.18	3.39	0.79
10^2	5.42	3.20	2.22
10^2	6.13	3.97	2.16
10^1	5.65	4.36	1.29
10^1	4.86	4.92	-0.06
10^1	4.94	5.00	-0.06
10^1	6.04	3.74	2.30
10^1	6.80	3.48	3.32
10^1	4.42	5.53	-1.11
10^1	6.02	3.55	2.47
10^1	5.73	3.32	2.41
10^0	3.59	3.93	-0.34
10^0	5.58	4.65	0.93
10^0	4.72	4.43	0.29
10^0	5.70	4.71	0.99
10^0	4.91	5.14	-0.23
10^0	4.88	4.47	0.41
10^{-1}	4.55	4.33	0.22

*Plaque Forming Units

TABLE 6

PIA SENSITIVITY DETERMINATIONS WITH THE ECHOVIRUS AS DETERMINED BY MEAN CLUMP SIZE
(2nd Series)

Echovirus Concentration PFU/ml *	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^3	3.77	3.63	0.14
10^3	3.40	4.60	-1.20
10^2	5.89	4.12	1.77
10^2	6.50	3.72	2.78
10^2	5.00	3.77	1.23
10^2	5.80	3.50	2.30
10^2	6.22	4.00	2.22
10^2	5.81	3.52	2.29
10^2	5.42	4.23	1.19
10^1	6.02	4.42	1.60
10^1	6.07	3.67	2.40
10^1	7.40	3.80	3.60
10^1	5.19	3.70	1.49
10^1	5.58	4.17	1.41
10^1	5.71	3.99	1.72
10^1	5.06	3.76	1.30
10^1	5.63	4.45	1.18
10^0	5.35	3.70	1.65
10^0	6.79	3.96	2.83
10^0	5.10	3.85	1.25
10^0	6.00	3.40	2.60
10^0	5.20	2.93	2.27
10^0	4.97	3.77	1.20
10^0	5.08	3.37	1.71
10^0	4.74	4.31	0.43
10^{-1}	3.96	4.17	-0.21
10^{-1}	4.39	4.61	-0.22
10^{-1}	4.30	4.80	-0.50

*Plaque Forming Units

3.1.2.1 THE PROPAGATION AND CHARACTERIZATION OF ECHOVIRUS TYPE 9

Echovirus Type 9 seed was received in our laboratory as 14MK, dated 6/7/68. The titer was listed as 10^7 infectious units/ml. Passage 1 pools were prepared on monolayers of WI-38 cells and AGMK cells in 32 oz prescription bottles. A 10^{-2} dilution of seed was prepared in Hanks' BSS to infect 10 bottles of each cell type with 1 ml each. The bottles were incubated one hour and each was fed 50 ml of HBME with 10% fetal bovein serum. The virus was harvested after 72 hours incubation at 37°C when approximately 90% of each monolayer was destroyed.

The assay of $P_{1\ WI-38}$ and $P_{1\ AGMK}$ were conducted in homologous cell lines. The plaque technique was used for both assays. Monolayers of WI-38 and AGMK cells were prepared in 25 cm^2 Falcon plastic flasks. Ten-fold serial dilutions were prepared from each passage 0.1 ml per flask was innoculated in triplicate into the cell line in which the virus was grown. The flasks were incubated one hour at 37°C and then overlaid with Basal Medium Eagle's with Hanks' Salts (HBME) containing 1% agar and 2% fetal bovine serum. The flasks were incubated a total of 11 days and refed at the midpoint with more of the agar-medium mixture. The cells were stained with neutral red containing medium and plaques scored after an additional 24 hours of incubation. Titers were found to be 3.5×10^5 PFU/ml for P_1 WI-38 and 3.8×10^7 PFU/ml for $P_{1\ AGMK}$.

3.1.3 HERPESVIRUS HOMINIS

PIA sensitivity determinations were performed on concentrations of herpesvirus ranging from 10^4 - 10^1 PFU/ml. The incubation time selected for these experiments was identical with the previously mentioned systems. The results of these experiments are summarized in Figure 4 and Table 7. Figure 4 shows the average signal and control curves surrounded by their respective 1 sigma bandwidths. The correlation coefficient of 0.99 shows an excellent dose-response in the concentration range of 10^2-10^{-2} PFU/ml. The intersection of the lower band of the signal curve and upper band of the control curve occurs at 0.6 PFU/ml, a measure of the sensitivity of the system.

Table 7 again shows the possible effects of a prozone-like phenomenon or saturation effect at high agent concentrations that was previously discussed. Table 8 shows the results of the individual experiments used in the PIA sensitivity measurements of the herpesvirus. The average control value of 4.0 with a standard deviation of 0.37, reflect a good reproducibility in the herpesvirus system.

3.1.3.1 THE PROPAGATION AND CHARACTERIZATION OF HERPESVIRUS HOMINIS

A CAM pool of herpesvirus was produced by infecting the CAM of 12-day old embryonated hen's eggs with 0.1 ml of a 10^{-2} dilution of the herpesvirus seed. The eggs were incubated for 48 hours at 35°C. The virus was harvested by removing the membranes and grinding them in a tissue grinder to a 20% (V/V) suspension. The debris was removed by sedimentation at 5,000 rpm in a refrigerated SS34 Servall centrifuge for 10 minutes.

Pools of herpesvirus were prepared in CEF and AGMK cells by infecting three 32 oz bottles of each cell type with 0.1 ml of the herpesvirus seed. The bottles were incubated for one hour at 37°C; the cells were fed with 50 ml each of Basa Eagles medium in Hanks' balanced salt

Average PIA Sensitivity Determinations with the Herpesvirus as Determined by Mean Clump Size

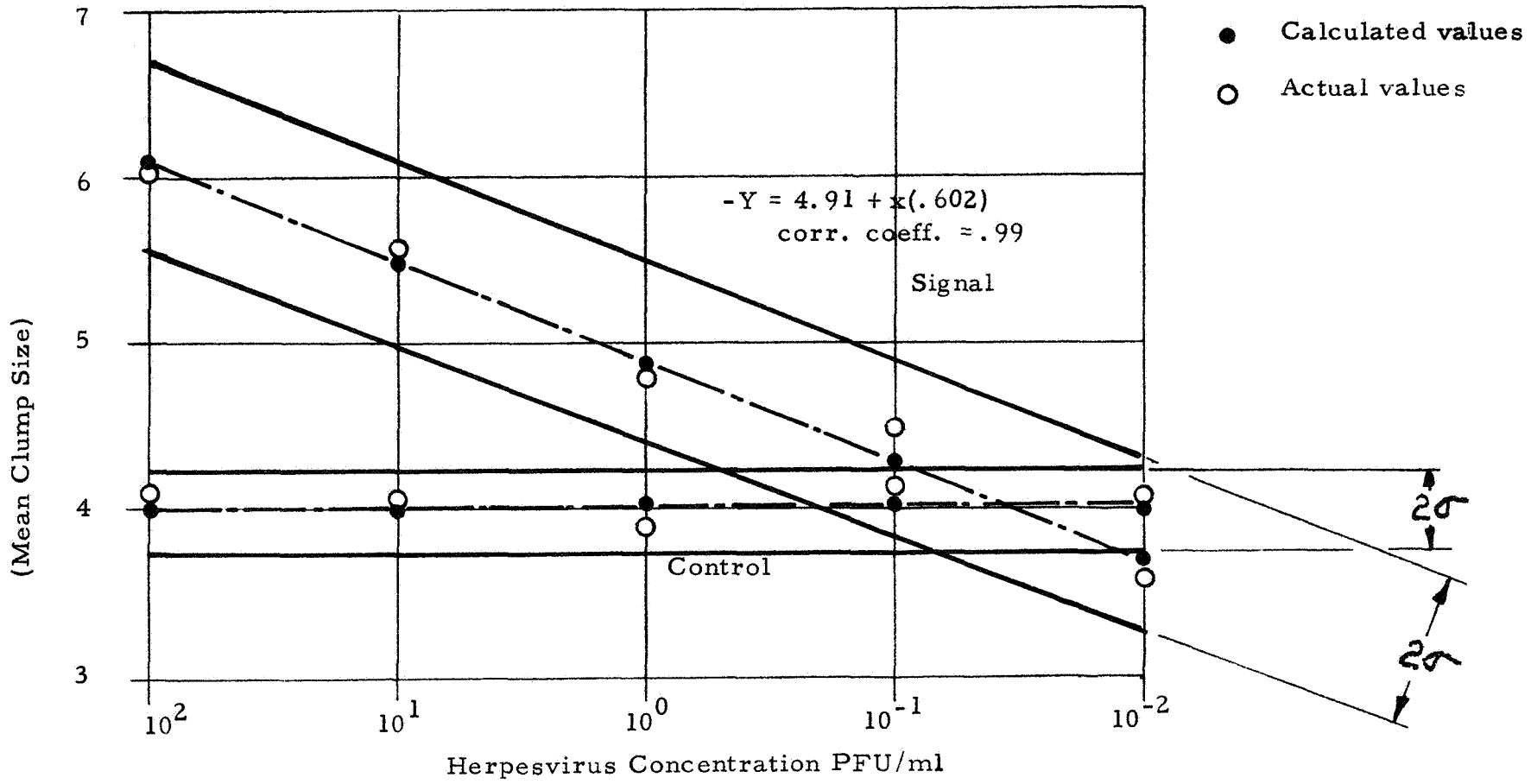


Figure 4

Table 7

AVERAGE OF VARYING CONCENTRATION OF THE HERPESVIRUS AS MEASURED BY MEAN CLUMP SIZE

Herpesvirus Concentration PFU/ml*	Signal (S)	Noise (N)	S-N
10^4	4.90	3.83	1.07
10^2	6.06	4.20	1.86
10^1	5.63	4.06	1.57
10^0	4.80	3.90	0.90
10^{-1}	4.48	4.23	0.25
10^{-2}	3.62	4.10	-0.48

Average Noise = 4.0
Standard Deviation = 0.37

*PFU - Pock Forming Units/ml

TABLE 8

PIA SENSITIVITY MEASUREMENTS OF THE HERPESVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Herpesvirus Concentration PoFU/ml*	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^4	4.80	3.91	0.89
10^4	5.03	3.99	1.04
10^4	4.87	3.59	1.28
10^2	5.21	3.87	1.34
10^2	5.14	4.19	0.95
10^2	7.79	4.48	3.31
10^2	6.17	4.28	1.89
10^2	5.99	4.16	1.83
10^1	4.82	4.69	0.13
10^1	4.81	3.88	0.93
10^1	6.20	4.08	2.12
10^1	6.47	4.03	2.44
10^1	5.86	3.62	2.24
10^0	5.04	3.76	1.28
10^0	4.44	3.62	0.82
10^0	5.17	3.99	1.18
10^0	4.58	4.22	0.36
10^{-1}	4.81	4.23	0.58
10^{-1}	4.15	4.44	-0.29
10^{-1}	4.49	4.01	0.48
10^{-2}	3.62	4.15	-0.53

*Pock Forming Units

solution (HBME). The infected cells were incubated 48 hours at 37°C and observed after 24 hours for cytopathic effect (CPE). After 48 hours most of the monolayers were destroyed or showed gross CPE. The fluid from these cytopathized cultures was harvested and assayed separately on homologus cell monolayers.

The herpesvirus harvests were assayed by the plaque technique of Dulbecco and by the pock method of Burnet and coworkers where applicable.

The herpesvirus grown on the CAM was made into a 20% suspension as already described. It was assayed on CAM. An aliquot of the suspension was used to make 10-fold serial dilutions. From each dilution a 0.05 ml aliquot each was innoculated onto the CAM of six 12-day old embryonated hen's eggs. After incubation at 37°C, the membranes were floated in saline and the pocks were counted and end-points determined following Armitage's method of analysis. Pock forming units (PoFU) per ml were then calculated. Herpesvirus P_1 was assayed on CAM by the pock technique with a resultant titer of 2.5 x CAM 10^6 PoFU/ml.

The harvests from CEF P_1 and P_2 and AGMK P_1 were assayed fro plaque forming units (PFU). Monolayers of CEF in 250 ml plastic Falcon plastic tissue culture flasks were used. Ten-fold serial dilutions were prepared from the tissue culture harvest. Flasks were innoculated in triplicate with 0.1 ml each per dilution. The flasks were incubated for 1 hour at 37°C, then overlaid with HBME containing 1% agar. The flasks were then incubated for 48 hours and stained with an overlay of HBME with 1% agar containing neutral red 1:40,000, final concentration. After 24 hours of staining, the plaques were scored and titers calculated.

Plaques stand out on a neutral red stained monolayer background as clear unstained circular areas. The number is directly proportional to the amount of live virus present in the inoculum since the agar overlay restricts the spread of the virus. Using this technique P_1 and P_2 CEF were found to contain 4.6 x 10^6 and 4.7 x 10^6 PFU/ml respectively.

3.1.4 ADENOVIRUS, TYPE 4

PIA sensitivity determinations were performed on the adenovirus under experimental conditions identical with the herpesvirus experiments. The concentrations of the adenovirus tested ranged from 10^4 to 10^{-3} $TCID_{50}$/ml with a useful detection range of 10^4 - 10^{-1} $TCID_{50}$/ml. Figure 5 shows the average signal plotted against agent concentration.

This figure shows that the dose response of the adenovirus was linear only for concentrations of 10^{+1} to 10^{-1}. This very narrow dynamic range plus a sensitivity of 3-5 $TCID_{50}$/ml can be directly attributable to the low complement fixation titer (1:40) of the antibody received from NASA. The low-titered antibody also accounts for the prominent large concentration effect which can be seen from Table 9, together with a low average control of 2.7 and standard deviation of 0.33. This effect, which is a result of either antigen excess or scarcity of antibody, is shown by the lack of a dose response at adenovirus concentrations higher than 10^1 $TCID_{50}$/ml. The individual experiments that were performed are shown in Table 10.

An increase in antibody titer would not only extend the dynamic range of the PIA test for adenovirus but also increase the sensitivity by at least one order of magnitude.

Average Signal of PIA Sensitivity Determinations With the Adenovirus As Determined by Mean Clump Size

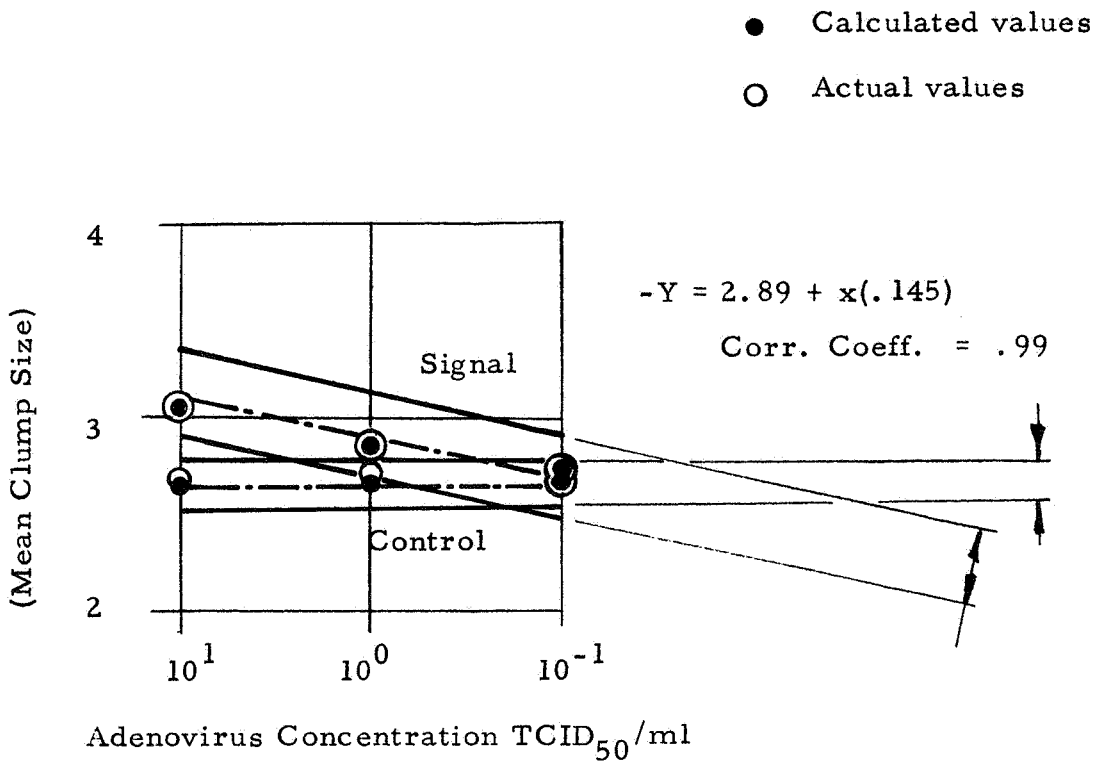


Figure 5

Table 9

AVERAGE PIA SENSITIVITY DETERMINATIONS WITH THE ADENOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Adenovirus Concentration $TCID_{50}$/ml*	Signal (S)	Noise (N)	S-N
10^7	3.42	2.76	0.66
10^5	3.12	2.92	0.20
10^3	2.89	2.61	0.28
10^2	2.92	2.76	0.16
10^1	3.03	2.65	0.38
10^0	2.88	2.86	0.02
10^{-1}	2.76	2.61	0.15

Average Noise = 2.72

Standard Deviation = 0.33

*TCID - Tissue Culture Infectious Dose

TABLE 10

PIA SENSITIVITY DETERMINATIONS WITH
ADENOVIRUS AS MEASURED BY MEAN CLUMP SIZE

Adenovirus Concentration TCID50/ml *	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^7	3.42	2.76	0.66
10^5	3.47	3.02	0.45
10^5	2.76	2.82	0.06
10^3	3.30	2.81	0.49
10^3	2.60	2.57	0.03
10^3	2.77	2.38	0.39
10^3	2.91	2.68	0.23
10^2	2.96	2.92	0.04
10^2	2.63	2.67	-0.04
10^2	2.37	2.25	0.12
10^2	2.94	3.00	-0.06
10^2	3.09	2.85	0.24
10^2	3.53	2.90	0.63
10^1	3.05	3.45	-0.40
10^1	2.25	2.23	0.02
10^1	2.64	2.55	0.09
10^1	2.92	2.33	0.59
10^1	3.17	2.49	0.68
10^1	2.61	2.40	0.21
10^1	3.67	2.71	0.96
10^1	3.96	3.05	0.91
10^0	2.03	2.06	-0.03
10^0	2.67	2.90	-0.23
10^0	2.63	2.77	-0.14
10^0	2.68	2.74	-0.06
10^0	2.35	2.69	-0.34
10^0	3.51	3.30	0.21
10^0	3.66	3.06	0.60
10^0	3.57	3.36	0.21
10^{-1}	3.02	3.06	-0.04
10^{-1}	2.83	2.58	0.25
10^{-1}	2.36	2.40	-0.04
10^{-1}	2.81	2.62	0.19
10^{-1}	2.68	2.90	-0.22
10^{-1}	2.35	2.08	0.27
10^{-1}	3.25	2.64	0.61

*Tissue Culture Infectious Dose

3.1.4.1 THE PROPAGATION AND CHARACTERIZATION OF ADENOVIRUS TYPE 4

Strain R1-67 seed adenovirus type 4 was received in our laboratory in the frozen state with the passage history of H19KB8.

Adenovirus was propagated on monolayers of H-Ep-2, HeLa and AGMK. One ml of a 10^{-2} dilution aliquot of the seed virus was innoculated into three 32 oz prescription bottle monolayers of H-Ep-2, HeLa and AGMK cells. The bottles were incubated 1 hour at 37°C, and then few with HBME (50 ml each) containing 10% (V/V) fetal bovine serum. The infected cells were incubated at 37°C for 48 hours. The virus suspension was harvested and assayed by determining the highest dilution of the harvest fluids which produced degeneration in 50% of the cell cultures infected, the 50% tissue culture infective dose ($TCID_{50}$). Each harvest was diluted in serial 10-fold dilution with HBSS. Four 13 x 125 mm screw cap tubes of AGMK monolayers were infected per dilution for each harvest. Each tube received a 0.05 ml aliquot of the proper virus suspension. After incubation for one hour, all tubes were fed with 2.5 ml of HBME (containing 10% serum) and incubated for 48 hours and scored for CPE. The titers were calculated by the Reed-Muench method. The results are as follows:

P_1HeLa	1.4×10^6 $TCID_{50}$/ml
P_1 H-Ep-2	1.0×10^5 $TCID_{50}$/ml
P_1 AGMK	1.5×10^8 $TCID_{50}$/ml

3.1.5 MYXOVIRUS A_2 INFLUENZA (HONG KONG) VIRUS

PIA sensitivity determinations were performed with the myxovirus and with beads sensitized with anti-myxovirus antibody that was made in our laboratories (CF titer of 1:1000).

Reaction conditions for the test were identical to those of the other tests. Figure 6 shows the signal plotted against agent concentrations with a 1 sigma bandwidth around the control and signal values. This figure shows the sensitivity of th system to be approximately 10^{-1} EID_{50}/ml. The correlation coefficient for the linear best fit through the signal points is 0.87 which shows a good dose-response.

The sensitivity of the PIA test for the myxovirus can be determined from the intersection of the lower 1 sigma band of the signal and the upper 1 sigma band of the control curve. The sensitivity under these conditions is approximately 0.1 EID_{50}/ml. This sensitivity is well below that required; i.e., 1-10 EID_{50}/ml. The next step would be to increase the reliability of the test for clinical sample testing.

Table 11 shows that at virus concentrations higher than 10^2 a prozone effect was evident, as demonstrated by a lower signal at higher virus concentrations. This concentration effect was also evidenced with the other virus systems. This effect for the myxovirus should not cause serious problems in diagnostic work for it is highly unlikely that a 10^2 EID_{50}/ml mkxovirus concentration would exist in a clinical sample. Table 12 shows the results of the individual experiments that were performed with the myxovirus, with an average control value of 3.05 and standard deviation of 0.38 which is within expected limits.

AVERAGE SIGNAL DETERMINATIONS WITH THE MYXOVIRUS AS DETERMINED BY THE MEAN CLUMP SIZE

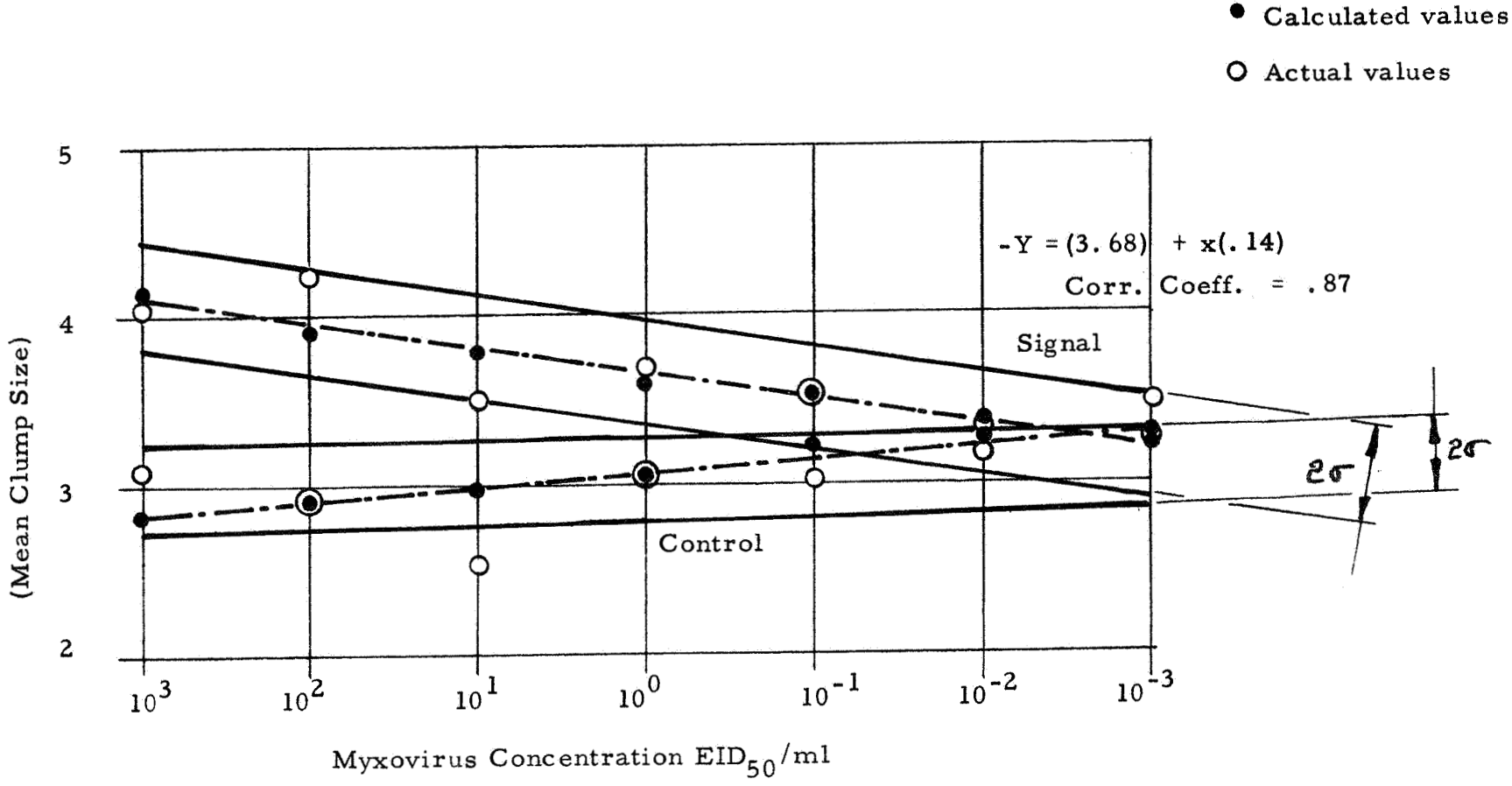


Figure 6

Table 11

AVERAGE SIGNAL OF VARYING CONCENTRATIONS OF THE MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration EID_{50}/ml*	Signal (S)	Noise (N)	S-N
10^3	4.05	2.71	1.34
10^2	4.20	2.98	1.22
10^1	3.52	2.54	0.98
10^0	3.77	3.19	0.58
10^{-1}	3.55	3.02	0.53
10^{-2}	3.32	3.33	-0.01

Average Noise = 3.05

Standard Deviation = 0.38

EID - Egg Infectious Dose

TABLE 12

PIA SENSITIVITY DETERMINATIONS WITH MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration EID_{50}/ml *	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^6	3.52	3.21	0.31
10^6	3.33	2.98	0.35
10^6	3.07	3.09	-0.02
10^5	3.77	2.81	0.96
10^5	3.97	2.95	1.02
10^4	2.86	3.06	-0.20
10^4	3.85	2.84	1.01
10^4	3.39	2.79	0.60
10^4	3.42	2.75	0.67
10^4	3.40	2.58	0.82
10^4	3.60	3.13	0.47
10^3	4.16	3.06	1.10
10^3	4.77	2.61	2.16
10^2	3.33	2.81	0.52
10^2	4.81	2.86	1.95
10^2	4.32	3.14	1.18
10^2	4.14	2.85	1.29
10^2	3.56	3.30	0.26
10^2	4.63	2.90	1.73
10^2	3.75	2.86	0.89
10^1	3.47	2.34	1.13
10^1	3.08	2.77	0.31
10^1	3.92	2.58	1.34
10^1	3.72	2.60	1.12
10^1	4.24	2.81	1.43
10^1	3.30	2.45	0.85
10^0	2.95	2.24	0.71
10^0	3.50	3.10	0.40
10^0	3.97	3.44	0.53
10^0	3.70	3.23	0.47
10^0	3.95	3.01	0.94
10^0	3.64	3.05	0.59
10^0	4.49	2.92	1.57
10^0	3.65	2.91	0.74
10^0	3.11	2.94	0.17
10^0	3.47	2.95	0.52
10^0	3.79	3.64	0.15
10^0	4.01	3.43	0.58
10^0	3.96	3.65	0.31
10^{-1}	3.67	3.18	0.49
10^{-1}	4.27	2.73	1.54
10^{-1}	3.88	3.25	0.63
10^{-1}	3.51	3.08	0.43
10^{-1}	3.94	2.97	0.97
10^{-1}	3.29	3.28	0.01
10^{-1}	3.49	3.39	0.10
10^{-1}	3.37	2.93	0.44
10^{-1}	2.50	2.38	0.12

*Egg Infectious Dose

TABLE 12 (continued)

PIA SENSITIVITY DETERMINATIONS WITH MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration EID_{50}/ml *	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
10^{-2}	3.65	2.94	0.71
10^{-2}	3.11	3.80	-0.69
10^{-2}	3.79	3.05	0.74
10^{-2}	3.49	3.50	-0.01
10^{-2}	3.04	3.49	-0.45
10^{-2}	3.28	3.78	-0.50
10^{-2}	3.92	3.79	0.13
10^{-2}	2.58	2.73	-0.15
10^{-2}	3.03	2.90	0.13
10^{-3}	3.10	3.73	-0.63
10^{-3}	3.72	3.28	0.44
10^{-3}	3.20	3.76	-0.56

3.1.5.1 PROPAGATION AND CHARACTERIZATION OF THE MYXOVIRUS

The Asian influenza virus (Hong Kong strain, egg passed only) was received in the lyophilized state. The specimen was reconstituted and three aliquots of 0.1 ml each were innoculated into the chorioallantoic sac of three 10-day old embryonated hens' eggs, 0.1 ml per egg. An aliquot of 0.1 ml was diluted by four 10-fold serial dilutions with Hanks' Balanced Salt Solution (HBSS), pH 7.2. An 0.1 ml aliquot of each dilution was innoculated into the chorioallantoic sac of 10-day embryonated hen's eggs in quadruple. The chorioallantoic fluid from each egg was harvested and stored separately after incubation at 35°C for 48 hours. Each harvest was tested for hemagglutination (HA) by mixing an aliquot of each with an equal volume of 0.5% chicken red blood cells (RBC). The harvests from dilutions 10^{-2} and 10^{-3} were positive for HA. A titration for HA activity demonstrated an end-point at 1:64 for harvests from the 10^{-3} dilution. A second passage, (P_2), of the 10^{-3} harvest diluted through 10^{-4} again gave a peak HA titer at the 10^{-3} dilution; this time the titer was found to be 1:128. The third passage increased the HA titer to 1:256 but a fourth passage did not increase hemagglutinating activity.

The harvest from passage 4 was assayed for infectivity. The titer, in 50% response egg infectious doses (EID_{50}), was found to be 1×10^6. Further passages of the myxovirus did not affect the HA titers but did elevate the EID_{50} to 1.6×10^8.

The first assay method used for the influenza virus was the hemagglutination reaction. The egg harvest, allantoic fluid, was diluted in doubling dilutions with 0.1 M phosphate buffered saline (PBS) in test tubes. Each dilution received an equal volume of 0.5% checken RBC. After mixing, the test was allowed to incubate 1-1/2 hrs at 22°C with observations for hemagglutination at 30 min intervals. Agglutination

signifies presence of the virus and non-agglutination signifies absence of the virus. The tube with the highest dilution giving agglutination is taken as the end-point.

A modified version of the HA test was also used to measure the amount of infectious virus present; i.e., the egg infectious dose (EID_{50}) titration method. The standard Reed-Muench technique was used to place the 50% response dilution.

The EID_{50} end-point assay of influenza virus was conducted by innoculating the chorioallantoic sac of 10-day old embryonated hen's eggs in quadruple with 0.1 ml of 10-fold serial dilutions of the virus suspension in question. The eggs were incubated at 35°C for 48 hrs. The chorioallantoic fluid was harvested; the yield for each egg kept separately. Tests for HA activity were conducted by mixing equal volumes of the individual harvest fluids with an equal volume of 0.4% human "O" RBC. The end-point of HA activity was read after 1-1/2 hrs at room temperature. The end-point was taken at the highest dilution showing HA activity in 50% of the eggs infected. The Reed-Muench method was used to calculate the EID_{50}.

Passage 3 was the first pool size harvest of the influenza virus with a sufficiently high titer to provide meaningful information on aging. Over a six-month period P_3 showed essentially no loss of HA or EID_{50} activity stored at -65°C as the raw harvest fluid.

3.1.6 MYCOPLASMA PHEUMONIAE

Initial sensitivity tests with Mycoplasma pneumoniae showed that the PIA detection sensitivity was approximately 10^5 - 10^4 CFU/ml (colony-forming units). At this level the signal was still marginal. The marginal tests were run with beads sensitized with NASA-supplied antibody that had a CF (complement fixation) titer of 1:16. It was believed at the time that one explanation for the low sensitivity could be the low titer of the antibody used to sensitize the beads. Consequently, rabbits were placed on an immunization schedule with the mycoplasma antigen. (See Section 3.1.5). The CF titer of the resulting immune serum was 1:1000. This serum was fractionated with 1/2 saturated ammonium sulfate and then with 1/3 saturated ammonium sulfate. This fractionated serum was then used to sensitize 1.1 μ polystyrene latex beads. Sensitivities of the order of 10^3 CFU/ml were obtained with the newly sensitized beads. Initial experiments were performed in which the sensitized bead stock was diluted 1:5 before it was reacted with the antigen. The results from these experiments are shown in Figure 7. In an effort to improve the sensitivity, the bead stock was not diluted before it was reacted with the antigen. The results of these tests are also shown in Table 13 &14. The average control values of 2.39 with a standard deviation of 0.36 demonstrate a reasonable fluctuation for the number of samples run.

The stability of the bead/antibody bond not only determines the longevity of the sensitized bead but also the maximum sensitivity that can be obtained with this particular reagent. Fluorescein isothiocyanate-tagged (FITC) rabbit anti-Bovine serum albumin (BSA) was heat fixed on 1.1 micron polystyrene beads in the usual manner. The amount of tagged antibody that dissociated from the beads during one hour incubation at 40° C was measured by removing the beads from the solution by centrifugation and measuring the fluorescence in an Aminco Bowman spectrometer. The amount of protein that was found in the supernatant was approximately 10 μg/ml. This amount is equivalent to 10^{13} antibody molecules/ml. This amount of antibody molecules will neutralize about 5×10^{12} BSA molecules/ml. This quantity of BSA is also equivalent to 10^5 CFU/ml. However, not all the protein that is removed from the bead is antibody, which accounts for the higher than expected sensitivities. The soluble antibody binds the antigen in solution forming soluble antigen-antibody complexes that are never detected because the antibody is

AV AGE SIGNAL WITH MYCOPLASMA PNEUMOI E AS
DETERMINED BY MEAN CLUMP SIZE

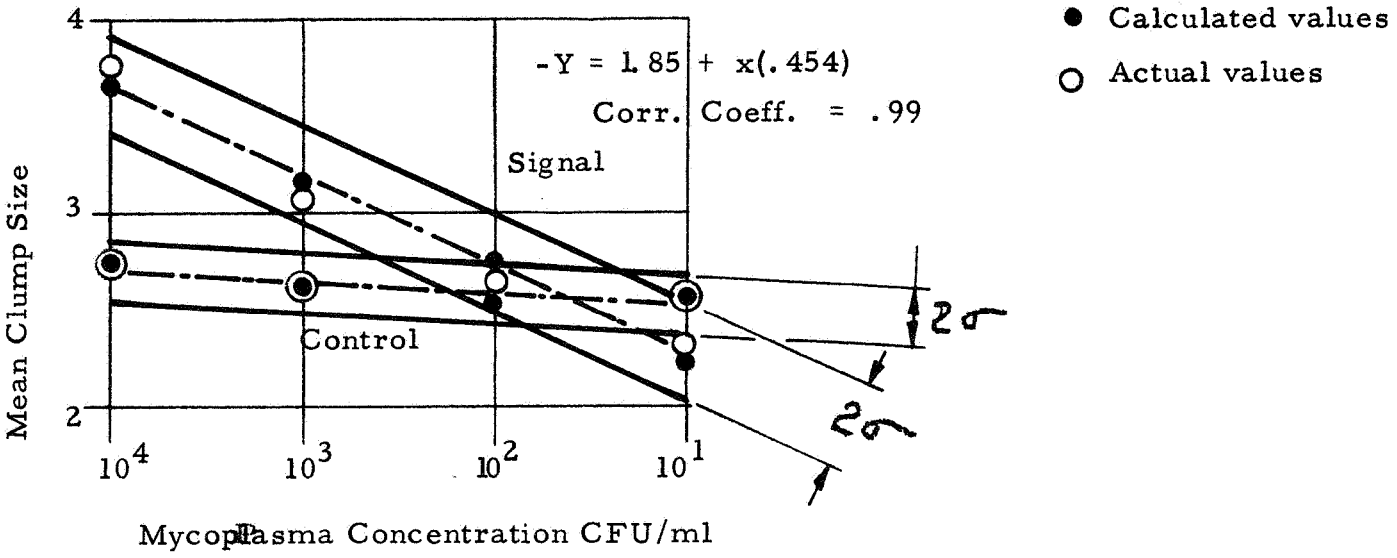


Figure 7

Table 13

AVERAGE S-N OF PIA DETERMINATIONS WITH THE MYCOPLASMA AS DETERMINED BY MEAN CLUMP SIZE

Mycoplasma Concentration CFU/ml *	Signal (S)	Noise (N)	S-N
10^4	3.71	2.91	0.80
10^3	3.09	2.49	0.60
10^2	2.66	2.57	0.09
10^1	2.40	2.64	-0.34

Control Average = 2.39

Standard Deviation = 0.35

*CFU = Colony Forming Unit

Table 14

PIA SENSITIVITY DETERMINATIONS OF THE MYCOPLASMA AS MEASURED BY THE MEAN CLUMP SIZE

Mycoplasma Concentration CFU/ml *	Mean Clump Size Signal (S)	Noise (N)	S-N	Bead Stock Dilution
10^6	2.23	1.70	0.53	1:5
10^5	2.43	2.02	0.41	1:5
10^4	2.42	2.07	0.35	1:5
10^3	2.36	2.11	0.25	1:5
10^5	3.00	2.24	0.76	1:0
10^4	3.71	2.91	0.80	1:0
10^3	2.69	2.37	0.32	1:0
10^3	3.50	2.62	0.88	1:0
10^2	2.98	2.93	0.05	1:0
10^2	2.32	2.55	-0.23	1:0
10^2	2.68	2.22	0.46	1:0
10^1	2.23	2.57	-0.34	1:0
10^1	2.58	2.71	-0.13	1:0
10^0	2.45	2.57	-0.12	1:0

Control Average = 2.39

Standard Deviation = 0.35

*CFU = Colony Forming Unit

not attached to a bead.

Therefore, the theoretical maximum mycoplasma sensitivity that one can achieve with a heat-fixed antibody by the present procedure appears to be 1000-500 CFU/ml. This is the limit that has presently been achieved.

To achieve the maximum sensitivity of 1-2 CFU/ml, it would be necessary to prevent the slow dissociation of the antibody from the bead. There are cases in the literature where antibody has been covalently bound to a solid substrate. Unfortunately most of the substrates that have been used have either been of amorphous character or much larger than the 1.1 micron sphere presently in use. Cellulose polymers have been used with reasonable success to covalently bind proteins. Sephadex G-25 superfine particles were used to determine if a cellulose polymer could be used to solidly fasten antibody in our case. These particles were 10-40 microns in size. Unfortunately, beads of smaller size were not commercially available so a PIA test in its present form was impossible. Nevertheless, it was possible to test the binding capacity of activated superfine beads.

It became apparent after the initial runs that increased antibody titer is only a partial solution to the problem of low PIA sensitivities related to the sensitivity with mycoplasma antigen. Two additional factors play an important role in the overall sensitivity of th system. One is the size of the antigen and the second is the quality of the sensitized bead in terms of the stability of the bead/antibody bond.

The size of the antigen for the PIA can be at least as small as a BSA (bovine serum albumin) molecule of 66,000 MW. By empirical measurement at 280 millimicrons, each mycoplasma antigen is the equivalent of 10^7 BSA molecules (per CFU).

This means that there are at least 10^7 antigenic molecules available for attachment in one CFU if the mycoplasma antigen can be broken to that size range before reaction with sensitized beads. Theoretically, one mycoplasma CFU could be amplified in terms of the PIA test 10^7 times. The theoretical amplification will probably never be realized. However, initial evidence shows that freezing and thawing a suspension two or three times will increase the sensitivity by 50%. The optimum method of mycoplasma breakage would yield sufficient antigenic particulates without denaturation

of the antigen itself. Freezing and thawing would be cumbersome in an automated system. However, such techniques as sonication and chemical lysis are methods that have been used for the disruption of other microorganisms, and should therefore be applicable to the mycoplasma problem as well.

FITC-labeled gamma globulin attached to the Sephadex beads in approximately the same quantity as obtained using the heat fixation procedure. However, upon washing, the amount of protein that was released into the supernatant was one tenth the amount that was released from the heat-fixed particles. This method shows great promise for permanently binding the antibody to the beads and will be tested thoroughly as soon as 1-5 micron beads become commercially available. This antibody binding technique promises to increase the sensitivity of the PIA test for mycoplasma by at least one order of magnitude, which combined with breakage of the mycoplasma itself, should give the necessary sensitivity of 1-10 CFU/ml.

3.1.6.1 THE PROPAGATION AND CHARACTERIZATION OF MYCOPLASMA PNEUMONIAE

A frozen isolate of Mycoplasma pneumoniae was received in our laboratory from NASA. The organism were grown in liquid cultures consisting of Difco PPLO medium fortified with 20% agamma horse serum and 2.5% yeast extract. A liter flask containing 500 ml of the fortified medium was innoculated with 3 ml of the seed. At time zero and each 24 hours thereafter, aliquots of the culture were taken and plated on fortified Difco PPLO agar. The plates were incubated under aerobic conditions at 37°C. Colonies were scored in 7-10 days with the aid of a dissecting microscope. A growth curve was plotted so that it would be possible to harvest the organisms during their most active growth stage, which typically is near the end of the logarithnic phase. The growth peak occurred between 192 and 216 hours. The end of the log phase occurred at 168 hours. All M. pneumoniae utilized in the study were harvested at 168 hours (See Figure 8).

For assay, serial 10-fold dilutions in Difco PPLO broth were prepared from an aliquot of the culture. Falcon plastic petri dishes, 60 mm diameter, containing fortified Difco PPLO agar were innoculated in triplicate with 0.1 ml from each dilution. The plates were incubated at 37°C and scored in 7-10 days. The titer was calculated from the colonies counted; i.e., colony forming units (CFU) per ml. The pool assayed at 9×10^5 CFU/ml. The 500 ml pool of M. pneumoniae was concentrated 50-fold by centrifugation at 16,000 g for 60 min. The drained pellet, except for a small aliquot in 10 ml of phenolized (0.05% phenol) Veronal buffer. The concentrate was titered at 10^8 CFU/ml when assayed on Difco PPLO agar. A total of 2.5 liters of M. pneumoniae was produced and concentrated to 5 ml volume.

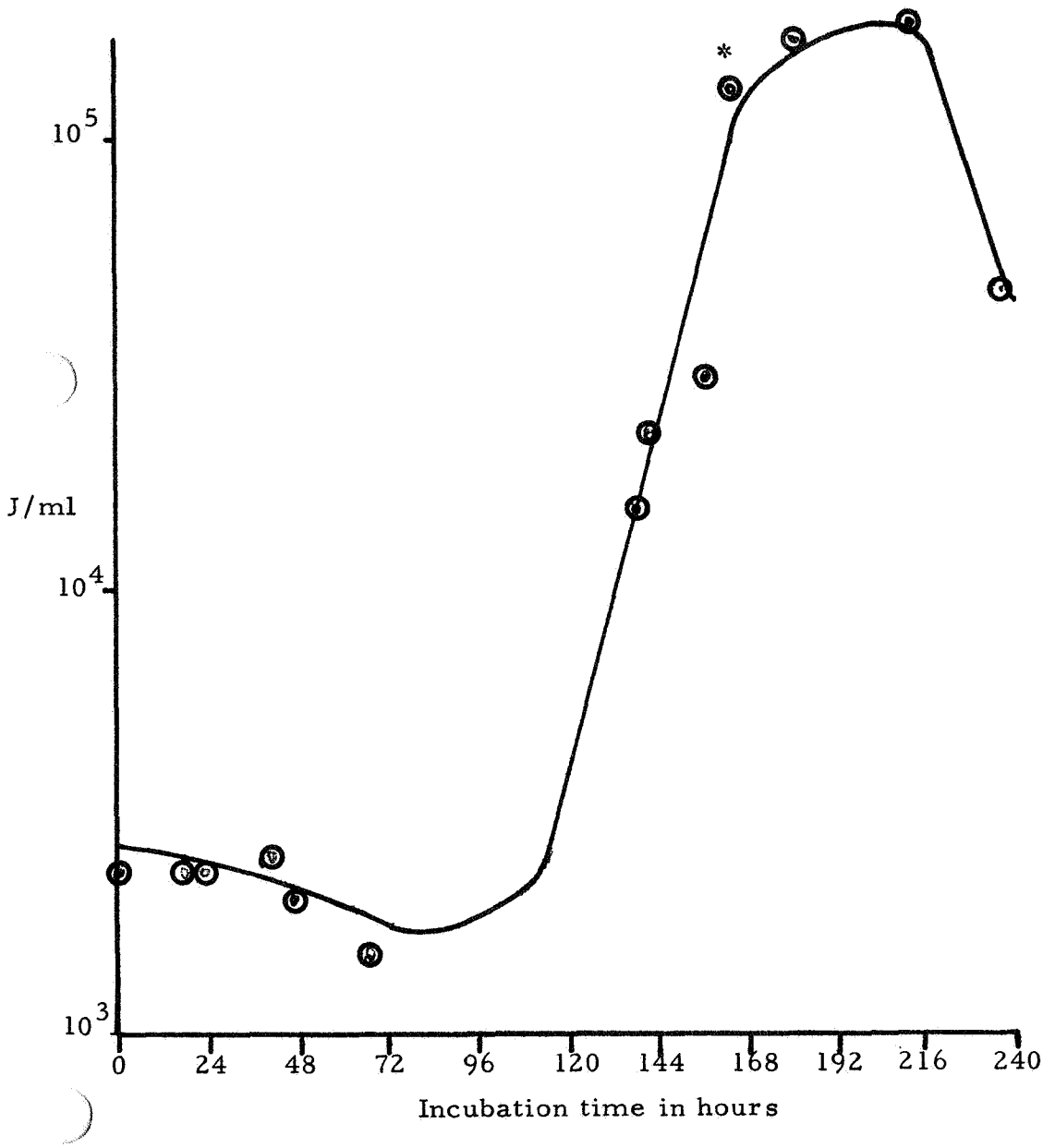


Figure 8

Growth Curve of Mycoplasma pneumoniae grown at 35°C in broth media. * notes time of harvest for use as antigen (168 hrs.).

3.2 SPECIFICITY OF THE PIA TEST

The usefulness of a specific detection system is only as good as its specificity. If the system is highly specific, identifications can be performed with a high degree of accuracy. A specific system of this type can also be made more general by either mixing sensitized beads of different specificities together or changing the specificity of the antibody globulin before the beads are sensitized.

Table 16 shows the average S-N values of three heterologous agents reacted with each of the six different sensitized bead preparations. In this table anything over +0.3 is considered a positive response. This table shows that there was negligible cross reactivity among the antigens tested. There was some scatter in the results of the individual experiments due to different antibody sources and different suspending fluids for the antigens. Nevertheless, the results demonstrate that the specificity of the PIA system for the agents tested is within acceptable limits.

3.3 ANALYSIS OF CLINICAL SPECIMENS

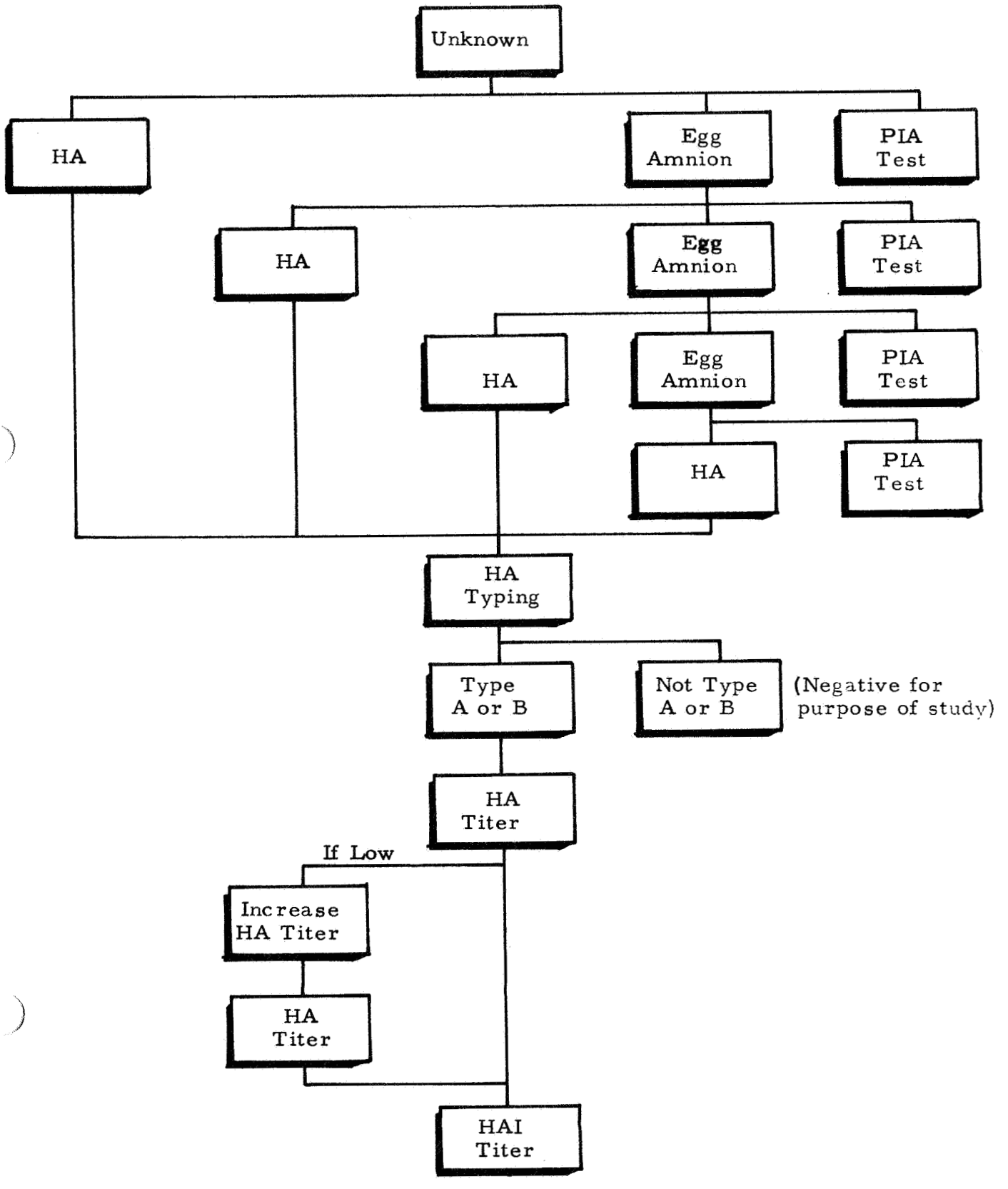
The feasibility of the PIA system was examined with regard to its effectiveness in detecting virus in clinical specimens. The advantage of testing clinical specimens with the PIA system is that the response time of 1-2 hours for the PIA test is far shorter than that required for more conventional methods (1-14 days). This rapid response time and high sensitivity allows direct examination of the specimen without growth amplification.

Eleven clinical specimens were examined both with conventional techniques and with the PIA technique. Six throat swab specimens were obtained from the Communicable Disease Center (CDC) in Atlanta, Georgia through NASA, and five came directly from NASA. The clinical samples from CDC were throat swabs that had been placed in tryptose phosphate broth. The other samples were in Hanks' BSS, physiological saline, or allantoic fluid. The conventional method for handling the clinical specimen is shown graphically in Figure 9 and explained in Section 3.3. The PIA procedure for handling the specimens was identical with the usual procedure performed in the laboratory.

Table 15

CROSS REACTIVITY DETERMINATIONS USING AVERAGE SIGNAL MINUS NOISE OF MEAN CLUMP SIZE

Agent Concentration		Sensitized Beads: Mycoplasma	Myxovirus	Adenovirus	Herpesvirus	Coxsackievirus	Echovirus
Mycoplasma	10^3 CFU/ml	**+.44**	-.07	+.26	-.13	+.13	-.38
Myxovirus	10^3 EID_{50}/ml	-.18	**+1.14**	-.08	-.63	-.19	-.48
Adenovirus	10^2 $TCID_{50}$/ml	--	--	**+.73**	--	--	--
Herpesvirus	10^2 PFU/ml	--	--	--	**+.44**	--	--
Coxsackie-virus	10^2 ID_{50}/ml	-.16	+.07	-.40	-.84	**+1.21**	+.01
Echovirus	10^2 PFU/ml	.03	-.24	--	--	-.56	**+.62**



CLINICAL SAMPLE TREATMENT PROCEDURE

The specimens from CDC were approximately one year old. There was some difficulty in handling these specimens in the PIA test. There were two reasons for this difficulty. One reason was the age of the specimen (~1 year), and the second reason was the tryptose broth suspending fluid of the sample. The PIA test is not normally conducted in tryptose broth because it is advisable to eliminate as much protein and protein by-products as possible from the reaction mixture. The excess protein material tends to interfere with the PIA reaction, and as a result 10-fold dilutions were made of the six samples for testing.

The control fluid used for the clinical specimen examinations was the suspending fluid of the sample being tested. The signal minus noise (S-N) from the four positive specimens was low. Part of the reason for this low signal was the 10-fold dilution at which the first six samples were run. This dilution reduced the number of virus particles available for detection. The other reasons for the low specific signals have already been discussed, i.e., the age of the sample and the suspending fluids. It is felt that the PIA test can be modified to utilize a suspending medium such as tryptose phosphate broth. However, the age of the sample will always be an important factor as numerous chemical and biological changes affecting antigenicity can occur even in biological samples that are frozen.

The five clinical specimens obtained from NASA were fresh and were suspended in Hanks' BSS, physiological saline, or allantoic fluid. No unusual effects from these samples, such as those that had occurred with the first six, were noted. The results obtained with all the clinical specimens are shown in Table 16

The data in this table indicate that out of the first six specimens received, three were positive, one weakly positive and two negative. Of the specimens received from NASA, 3 specimens gave a +4 reaction, one a +3 and one a +2 reaction. Table 17 shows the actual signals obtained from the samples. The first six samples shown gave much lower signals than did the last five. The reasons for the higher signals apparently related to the date of dampling of the sample and the fact that samples 7-11 were tested undiluted. Samples 1-9 were analyzed to fulfill contractual requirements and samples 10 and 11 were examined by oral request. Sample 9 gave the lowest signal of the 5 samples sent by NASA.

Table 16

PIA EXAMINATION OF CLINICAL SAMPLES

Date of Sample	Source	Unknowns	Dilution for test	Reaction* Intensity	Signal-Noise Mean Clump Siz
1-3-69	CDC 1	GSP #1	1:10	+2	0.26
1-3-69	CDC 2	GSP #2	1:10	+3	0.70
1-21-69	CDC 3	A-44	1:10	+4	0.80
1-21-69	CDC 4	A-51	1:10	±1	0.09
3-12-69	CDC 5	A-68	1:10	±1	0.05
3-17-69	CDC 6	A-62	1:10	+3	0.50
2-70	NASA 7	A	undiluted	+4	0.93
2-70	NASA 9	C	undiluted	+2	0.62
2-70	NASA 8	D	undiluted	+4	1.15
2-70	NASA 10	F	undiluted	+3	0.82
2-70	NASA 11	G	undiluted	+4	1.94

* Subjective evaluation of the agglutination reaction based on a 0-4 scale. (0 indicates no agglutination, while +4 indicates a dramatic difference from control).

Table 17

PIA DETERMINATIONS OF UNKNOWN CLINICAL SAMPLES

Sample Designator	Signal (S)	Noise (N)	S-N
6SP#1	2.76	2.50	0.26
6SP#2	3.30	2.60	0.70
A-44	3.25	2.45	0.80
A-51	3.34	3.25	0.09
A-68	3.54	3.49	0.05
A-62	3.21	2.71	0.50
A	3.36	2.43	0.93
C	3.00	2.38	0.62
D	3.77	2.62	1.15
F	3.05	2.23	0.82
G	4.22	2.28	1.94

This low signal relative to those obtained from the rest of the samples probably indicates one of two things: (1) the virus was in lower concentration than other samples; or (2) the virus was not the exact one that was being sought but is closely-related antigenically. The latter reason appears at present to be the best explanation. Virus was isolated from all the samples, and good HA titers could be obtained from the original samples, 7-11, as well as from the allantoic fluid passage harvest. Sample 9, however, gave a low HAI compared to the HA titer. This would incicate that the virus that was isolated is not the Hong Kong A_2 variety but a closely-related virus such as Influenza B. The antibody used to sensitize the beads was prepared against Influenza Hong Kong A_2 but was not absorbed with other antigenically similar viruses to remove cross-reacting antibodies. For increased specificity at the group level, cross reacting antibodies should be removed from the antisera.

3.3.1 CHARACTERIZATION OF 11 CLINICAL SPECIMENS

3.3.1.1 ISOLATION OF INFLUENZA A_2 HONG KONG VIRUS FROM MOCK CLINICAL SPECIMEN

Preparatory work for protocol testing was conducted on a mock clinical specimen which was divided into two aliquots. One, the sample, was seeded with P_3 influenza virus to give a final concentration of approximately 5 EID_{50}/0.1 ml. The other, the control, received an equal volume of sterile saline. Aliquots of 0.1 ml from both the sample and control were injected into the amnion of 11-day embryonated hens' eggs. After 48 hours incubation at 35°C, the amniotic and chorioallantoic fluids from each egg were separately harvested. Half of the sample eggs were positive for HA in the amniotic and chorioallantoic fluids. All the control eggs were negative for HA in both the amniotic and allantoic fluids. The positive allantoic fluids were then typed with chick and human "O" RBCs. The results indicated that the isolates from the mock clinical specimen could presumptuously be classified as Type A or B but not C influenza virus. The HA test was positive at both 4°C and 22°C within both cell types. The HA results were then confirmed by a standard HAI test with antiserum prepared in our laboratories from P_3 of influenza virus A_2, Hong Kong. The serum was capable of inhibiting HA at a dilution of 1:2560. Control negative serum demonstrated no inhibition of HA activity. The HA activity of the positive control virus, P_3, was inhibited at 1:2560.

3.3.1. NASA CLINICAL SPECIMENS

Eleven clinical specimens were received in our laboratory, 6 in the frozen state, and 5 in wet ice. Six specimens arrived 1/22/70; the other five were received 2/17/70. The specimens were labeled as follows:

1/22/70		2/17/70	
#1	GSP #1 1-3-69	#7	A in Hanks' BSS
#2	GSP #2 1-3-69	#8	C in allantoic fluid
#3	VA A-44 1-21-69	#9	D in Hanks' BSS
#4	VA A-51 1-21-69	#10	F in PBS
#5	A-68 3-12-69	#11	G in PBS
#6	VA A-62 3-17-69		

One-tenth ml of each sample was tested for HA with 0.1 ml of 0.4% human "O" RBC. All were negative for HA activity. Aliquots of 0.3 ml each were then taken from each specimen and mixed with an equal volume of penicillin and streptomycin to give a final concentration of 400 units of penicillin and 400 ug of streptomycin/ml. Six 10-day embryonated hens' eggs were innoculated in the amnion with 0.1 ml of the treated specimen. After 48 hours of incubation, the amniotic and chorio-allantoic fluids of each egg were separately harvested. Each fluid was tested for HA activity. Only specimen #1 was positive in the amnion. The other harvests of each specimen were pooled, keeping the amniotic and chorio-allantoic fluids separate. Aliquots of 0.1 ml of each pooled specimen were injected into the amnion of 10-day embryonated eggs. Ten eggs were used for each pooled specimen. Upon harvest of the individual fluids from this passage, specimen #2, #3 and #4 were positive for HA in both fluids. Specimen #5 and #6 were recycled after pooling each one in 10-day embryonated eggs. The second blind passage of specimen #5 and #6 was also negative for HA.

Aliquots from the original #5 and #6 samples were prepared and injected into the amniotic cavity of six 10-day embryonated eggs each. When harvested after 48 hours, both specimen were positive for HA activity in the amniotic and chorioallantoic fluids.

The positive fluids were pooled for each specimen and aliquots of each were studied with the PIA technique. A presumptive typing was carried out on the isolates by conducting HA tests at 4 and 22°C with human "O" and chick RBCs. The results were as follows:

PRESUMPTIVE TYPING

	4°C		22°C	
Spec. No.	Chick	Human	Chick	Human
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	-	+	-	+

From the above, it is presumed that a type A or type B myxovirus was isolated from each of the unknown specimen; specimen No. 6 could only be type A. Confirmatory typing followed by HAI studies on the isolates was employed for identification.

Aliquots of each pooled specimen were taken for use in the study of HA activity. The titers were as follows using 0.4% human "O" RBCs.

Specimen No.	HA Titer (P_2)
1	1:4
2	1:4
3	1:16
4	1:4
5	1:160
6	1:256*

*Specimen was passed an additional time since its harvest titer was less than 1:20.

These titers were considered relatively too low to complete the desired study. The titers were increased by passing each isolate in the chorio-allantoic sac of four 10-day embryonated eggs each. The HA titers on P_1 of each isolate were as follows.

Specimen No.	HA Titer
1	1:128
2	1:32
3	1:32
4	1:64
5	Not Done*
6	1:256

*The titer of Specimen #5 was sufficiently high without further passage.

Using standard methods, four HA units of each isolate were prepared and used in an HAI study. Each isolate was run in duplicate. The following results were obtained:

HAI STUDY

Specimen No.	HAI Titer (confirmatory)
1	1:1280
2	1:640
3	1:1280
4	1:1280
5	1:1280
6	1:1280
Positive Control (P_3)	
Negative Control (allantoic fluid)	

The second series of 5 unknown specimen received from NASA (2-17-70) also included 3 control fluids. The specimen were labeled:

7A	in Hanks' BSS
8C	in allantoic fluid
9D	in Hanks' BSS
10F	in PBS
11G	in PBS
12B	Hanks' BSS
13E	PBS
14H	Allantoic fluid

The first three were necessary to complete the series of nine contractually required tests; the additional two were considered as ancillary, to be processed if time permitted. The last three tubes were control fluids.

Aliquots of all the unknowns in the second series, including the controls, were injected into the amniotic cavity of six 10-day embryonated hens' eggs. The amniotic and chorioallantoic fluids of each egg were harvested, kept separate and spot checked for HA activity. The five unknowns were positive; the control fluids were negative. Two blind passes were made on the control fluids; they remained negative through al passed. Amniotic and allantoic fluids pooled for each individual isolate were used for typing

Presumptive typing of the new isolates gave the following results:

PRESUMPTIVE TYPING

	4°C		22°C	
Specimen	Chick	Human	Chick	Human
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+

These results indicate that all the isolates could be either type A or type B. Confirmatory tests followed.

The HA titer was determined on the pooled individual amniotic and allantoic fluids from each new isolate.

Specimen	HA Titer
7	1:40
8	1:160
9	1:640
10	1:160
11	1:160
Positive Control	
P_3 (CA)	1:256

Four HA units of each isolate were used in the HAI test for confirmatory typing of each isolate. The serum used was from the same pool used with earlier isolates. The results of the HAI tests conducted in duplicate with the new isolates were:

Specimen	HAI Titers (confirmatory)
7	1:640
8	1:40
9	1:1280
10	1:640
11	1:2560
Positive Control P_3 (CA)	1:2560
Negative Control	0

The unknowns, except #8, all appeared to be type A influenza virus based upon the HA and HAI patterns. Unknown 8 may be questionable since its HAI confirmatory titer is so very low. A 100 EID_{50} dose of all of the unknowns were neutralized by 1:40 dilution of influenza A_2 anti-serum in embryonated hens' eggs.

3.4 PURIFICATION AND CONCENTRATION

The purification and concentration of the crude virus suspensions were intended to remove extraneous proteinaceous material and reduce the volume of the suspending medium. In processing the viruses used in this study, one general type of diaflo ultrafiltration membrane XM-100 was used. This is the recommended membrane for purification and concentration of viruses, globulins and macromolecules. The results of purification and concentration in Table 18 show that only two of the five organisms, echovirus type 9 and adenovirus, were purified and concentrated to any extent with the XM-100 membranes. Their infectivity increased by almost a $\log_{(10)}$ or better over that of the original; their protein content decreased significantly from the originals. The other three viruses were not purified; indeed they concentrated protein with relative increase in infectivity. The infectivity of Coxsackievirus was more than two $\log_{(10)}$ lower in the concentrate than in the starting material. The Coxsackievirus effluent was very nearly the same as that of the original homogenate. The protein content of the suspension after processing was higher than the starting material, while the protein content of the effluent was decreased. The infectivity of herpesvirus and influenza A_2 did not change appreciably as a result of processing; the protein content of both suspensions was increased significantly. The processed effluents of both suspensions had lower levels of protein than did the corresponding suspension.

While these studies did not result in extremely pure or considerable concentration, they do indicate that purification and concentration can be achieved with the technique. The studies indicate that purification and concentration of the viruses used can be achieved by using the proper size of filter membrane.

Table 18

PURIFICATION AND CONCENTRATI

Virus	Protein (mg/ml)				Titer (/ml)		
	Concentration	Before	Residue	Effluent	Before	Residue	Effluent
Herpesvirus hominis	15-fold	3.75	6.25	2.5	4.6×10^6 PFU	8.2×10^5 PFU	Not done
Coxsackie-virus Group A2	15-fold	50.00	200.0	25.0	$4.0 \times 10^6 ID_{50}$	$1.1 \times 10^4 ID_{50}$	$1.0 \times 10^6 ID_{50}$
Echovirus type 9	30-fold	3.75	15.0	2.0	3.5×10^5 PFU	1.1×10^6 PFU	Not done
Adenovirus type 4	15-fold	5.0	3.75	3.75	$1.4 \times 10^6 TCID_{50}$	$5.0 \times 10^8 TCID_{50}$	Not done
Influenza A_2 Hong Kong	15-fold	2.5	7.5	1.75	$1.6 \times 10^8 EID_{50}$	$2.3 \times 10^8 EID_{50}$	Not done

3.5 STABILITY STUDIES

The advantage of an inert carrier versus a biological carrier, such as a red blood cell, is the inherent stability of the former. The "shelf life" of the sensitized bead then becomes a function of the longevity of the antibody and the carrier/antibody bond, and not of the carrier system itself. Six batches of polystyrene beads each sensitized with antibodies to one of the six organisms under study were examined from the time of sensitization to the end of the present technical effort. Special consideration was given to the beads sensitized with myxovirus and mycoplasma antibodies which were tested at one-month time intervals. The other four batches of beads were tested at greater intervals.

The influenza and mycoplasma sensitized particles were stored at 4°C at a concentration of approximately 10^9 beads/ml. These beads were tested at intervals of approximately one month to determine the effect of storage on the ability of the bead to retain its initial activity. Figure 10 shows the effect of storage on the sensitivity of the bead as a percentage of its original activity. With the exception of the mycoplasma and the adenovirus sensitized beads, no significant difference in sensitivity were noticed after 7 months of storage. The reason for the decline in mycoplasma sensitivity is due in part to the nature of the antigen itself. The antigen used for testing was stored for an approximately equal length of time. The mycoplasma antigen (due to its greater mass) is subject to more time-related change than the viral antigen. The poor stability of the anti-adenovirus sensitized beads may be attributed to the poor quality of the original antisera. Based on sensitivity studies, some additional loss in sensitivity can be attributed to the slow loss of antibody from the bead which would tend to form soluble antigen/antibody complexes in the reaction mixture. These complexes would not be detected. However, additional testing of the sensitized bead, increased quality control in the preparation, plus a more rigorous attachment (through higher-temperature binding) of the antibody to the bead should increase the effective stability of the sensitized bead to at least one year.

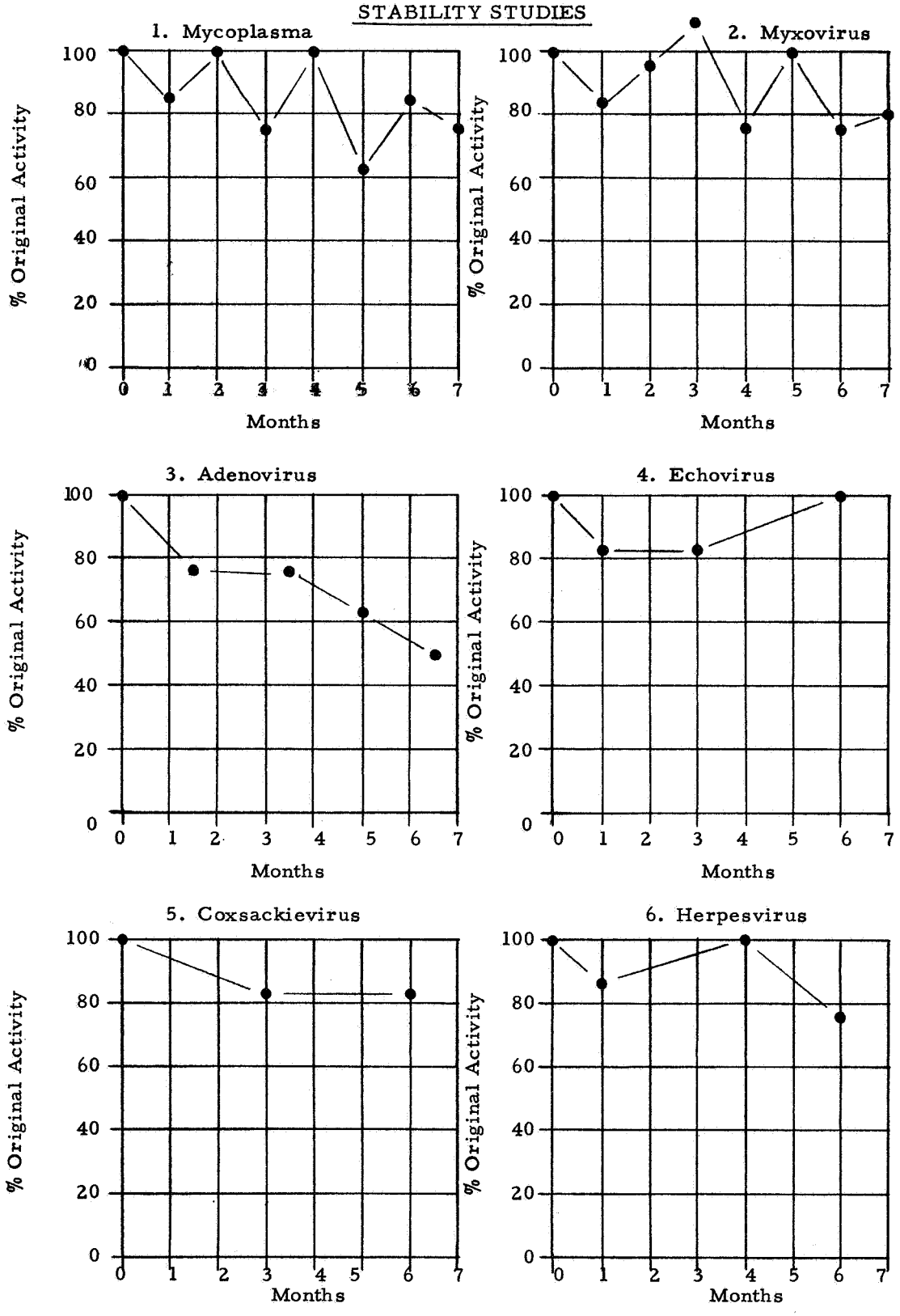

STABILITY STUDIES
1. Mycoplasma
2. Myxovirus
3. Adenovirus
4. Echovirus
5. Coxsackievirus
6. Herpesvirus
% Original Activity
Months
0
20
40
60
80
100
0 1 2 3 4 5 6 7

Figure 10

3.7 ANTIBODY PREPARATION AND CHARACTERIZATION

The antibody preparations received from NASA were characterized by the HAI or CF test. Diagnostic antisera specific for each test agent were obtained from Microbiological Associates. Use of these commercial antigens and antisera together with those supplied by NASA has allowed precise reagent baseline titrations, providing a reference point for each reagent with which future immunological reactions could be compared.

The data shown are averages of at least three tests. The titrations that were performed included hemagglutination (HA), hemagglutination inhibition (HAI) and complement fixation (CF) (See Appendix A for procedure). Table 19 shows results obtained when the diagnostic antigens for the myxovirus, adenovirus, herpesvirus and Mycoplasma pneumoniae were reacted with both the commercial antisera and with NASA-supplied antisera. In the case of the myxovirus, both the commercial antigen and antigen propagated in our laboratory from the NASA seed were reacted with the diagnostic antiserum, NASA antiserum, and a gamma globulin fraction of the GFE antiserum (see Appendix A for the fractionating procedure). The commercial antiserum was also cross-titered against both the diagnostic antigen and that propagated in our laboratory.

Table 19 also shows that when the sera were heat inactivated ($56^{\circ}C$ for 30 minutes), the CF titers were substantially reduced as expected. The observed decrease in CF titer upon heating does not, of course, indicate that the antibody activity has been lost. Indeed, the heat treatment is shown to have no effect on the PIA reaction.

The antisera to the six organisms under study were fractionated by a conventional ammonium sulfate precipitation technique (see Appendix A for procedure). Before salt fractionation, each serum was treated with equal volume of Freon 113 to remove many of the lipids

Table 19

STANDARDIZATION OF IMMUNE SERA

Antigen	Antigen Type	Test	Commercial Diagnostic Antibody	NASA Antibody		NASA Antibody
				Normal	Heat Inact. *	Fractionated
Influenza A_2 (Hong Kong)	Diagnostic	CF	1:64	1:64	Neg	Neg
Influenza A_2 (Hong Kong)	Diagnostic	HAI	1:128	-	-	-
Adeno 4	Diagnostic	CF	1:40	1:40	Neg	1:16
Herpes 9	Diagnostic	CF	1:128	1:128	1:16	1:128
Mycoplasma pneumoniae	Diagnostic	CF	1:128	1:16	Neg	1:16
	Laboratory Passage	CF	1:128	1:128	1:16	1:64
Coxsackievirus	Laboratory Passage	CF	-	1:128	-	-
Echovirus	Laboratory Passage	CF	-	1:128	-	-

*Heat inactivation was performed at 56°C for 30 minutes.

present in "raw" serum. A two-step fractionation is performed since previous studies have shown that the two-step technique provides an optimum sensitized latex preparation. The fractionated sera have been used to sensitize polystyrene latex beads by a heat fixation reaction.

Immunological schedules for Mycoplasma pneumoniae and influenza A_2 Hong Kong were set up to produce higher-titered, specific antisera for the PIA laboratory experimentation.

Injections were given to test rabbits on a weekly basis with a gradual buildup in agent concentration. The site and volume of the injections were dependent upon the reaction of the test animal to the antigen. At the end of seven weeks (seven injections), the rabbits were trial-bled and the raw serum was titered by a microtiter complement fixation (CF) method.

The Mycoplasma schedule, due to good titers and a time factor, was terminated after the seven-week period. The titers ranged from 1:256 to 1:1024 and were combined to form a pool having a titer of 1:512 (160 ml of fractionated antibody at a concentration of 7.96 mg/ml).

The myxovirus innoculated rabbits, at the end of seven weeks, produced CF titers averaging 1:100. The schedule was then continued for an additional three weeks using increased antigen concentrations and dual inoculation sites (I.M. and I.P. or I.M. and I.V.) to boost the titers. Sera from the test animals were re-titered by complement fixation. The titers ranged between 1:200 and 1:400. The sera were combined, fractionated and stored for use.

3.7 PIA SENSITIVITY DETERMINATIONS WITH STREPTOCOCCUS

The PIA test has been demonstrated to be a highly sensitive and specific test for viruses. The capability of this test can be extended to any system involving an antigen/antibody reaction. The only requirement necessary to perform a successful PIA test, assuming the test reagents are adequate, is that the antigen being detected be of relatively small size. The reason for this is that an antigen of 66,000 MW is sufficient to hold two sensitized beads together. If the antigen is as large as a bacterium, the probability is that it will still hold only two beads together. If the antigenic material on the bacterial cell is broken into smaller units, each antigenic unit is then capable of clumping tow particles. Therefore, each bacterium has the potential of holding large numbers of sensitized beads together, if the antigenic material can be removed from the cell proper.

The feasibility of the PIA test was examined for streptococcus detection, using 10^5 bacteria/test. The test was performed in an identical manner to the viral detection procedure. The whole bacteria gave no detectable signal even at the 10^5 bacteria/test level. A solution of the streptococci at the identical concentration was processed through a French press a single time. This single pass through the press resulted in complete chain breakage, and about 10% cell disruption as measured by cell counts. PIA tests run on the partially broken cells gave significant signals at 10^4 bacteria/test level. These results were obtained in a single series of experiments and indicate that if the bacterial antigenic material is amplified (by either mechanical or chemical breakage), higher sensitivities are possible. Additional study is clearly needed for the PIA test to be considered as a rapid, sensitive and specific method for detection of bacterial agents.

4.0 TECHNICAL DISCUSSION, THREE-MONTH CONTINUATION

The technical effort, comprising the three-month continuation, encompassed the following major areas: (1) improved antiserum production, (2) optimization of process variables - to include the effect of varying incubation conditions, ionic strength of the suspending fluids, bead concentration, bead size and sample mixing, (3) specificity testing, (4) examination of readout techniques for the PIA test, and (5) clinical specimen testing. This work was an extension of the first nine months' effort on the MEMS program and was designed to obtain additional information regarding reliability and reagent quality for dependable operation of the PIA test.

4.1 ANTISERUM PRODUCTION AND PURIFICATION

Earlier PIA determinations with the Mycoplasma indicated that the sensitivity of the test was dependent (among several other factors) on the quality of the antibody globulin attached to the latex beads. Therefore, attempts were initiated to prepare high-titered antisera to influenza A_2 Hong Kong virus and Mycoplasma pneumoniae. It was desired to produce a large enough quantity of the antisera to complete the three-month testing program. The utilization of a standardized reagent would prevent inconsistencies associated with this particular biological parameter.

The method for antigen preparation is described in Sections 4.2.1 and 4.2.2. For both agents, one goat and two rabbits were placed on the following immunization schedule. Weekly shots of the appropriate antigen were administered to the animals. After the third week, trial bleedings were taken (at weekly intervals) before each booster. The titers for the antisera against each organism are listed in Table 20

The titers of the antisera were determined by using antigen sensitized beads. The specific antigen is covalently bonded to the latex particles and reacted with various dilutions of the homologous antiserum. The titer of the particular antiserum is defined as the last dilution yielding complete agglutination. The conventional complement fixation titer (Table 21) is provided as a reference. The results indicate that the titer of the antisera against the Mycoplasma increased from 1:59,000 to 1:177,000. With the anti-influenza preparation, the titer remained the same (1:20,000). It is considered that the reactivity of both these antibody preparations will not

Table 20

ANTISERUM PREPARATION FOR MEMS

Antibody	Source	Titers	
Anti Mycoplasma	Commercial	< 3	(CF 1:8)
	Old "AMB" Rabbit Antisera	59,000	(CF 1:500)
	New "AMB" Rabbit and Goat Antisera	177,000	
Anti Influenza	Commercial	< 3	(CF 1:16)
	Old "AMB" Rabbit Antisera	20,000	(CF 1:500)
	New "AMB" Goat Antisera	20,000	

diminish the sensitivity of the PIA test. In addition, the production of sufficient quantities provides the capability to utilize a single preparation throughout the study, eliminating biological variations that would occur through the use of various preparations.

4.2 OPTIMIZATION OF PROCESS VARIABLES - SYSTEM SENSITIVITY

Various conditions of the PIA reaction which affect the overall sensitivity of the test were examined. A concentrated effort was placed on the time interval of incubation; however, bead size, reagent and sample mixing, bead concentration and the ionic strength of the suspending fluid were also studied.

The effect of bead size was evaluated with 1.01 μ, 1.10 μ and 2 μ sized beads. Initial results indicated that the 1.10 μ latex particles were most suitable (provided less nonspecific agglutination), consequently, this class of beads was used throughout the remainder of the study. Further evaluation, in light of new methods for sensitization or antigen pretreatment may indicate a reversal of this trend. The ionic strength of the suspending fluid (Glycine-Saline buffer) was evaluated by varying the concentration of both components. Results indicated that a 50% reduction in the concentration of both species reduced nonspecific agglutination. Bead concentrations in the reaction were varied from 10-50%, however, no conclusions can be made at this time of the effect on the PIA sensitivity. The sample and reagent mixing procedure was modified. The plastic reaction vessel was replaced with a 6 x 10 mm glass tube. The smaller size provides a greater probability for the specific reaction to occur, while reducing evaporation that may attach reacted beads to the sides of the vessel.

4.2.1 INFLUENZA A_2 HONG KONG VIRUS

Sensitivity Studies

The purpose of these studies was to examine the effect of varying incubation time intervals and the use of high-quality antibody on PIA sensitivity. The beads in this study were sensitized with the goat prepared antisera, fractionated and purified by the techniques described in Appendix A, and attached by a heat fixation reaction. Several lots of antigen were prepared and utilized for the evaluation. Table 21 represents the summarized data

Table 21

SUMMARY, PIA SENSITIVITY DETERMINATIONS WITH THE MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Concentration (EID_{50}/ml)	Signal (S)	Noise (N)	S-N
30 min. Incubation			
10^7	3.40	2.38	1.02
10^5	2.86	2.09	0.77
10^4	2.39	2.12	0.27
10^3	2.50	2.18	0.32
10^2	2.31	2.14	0.17
10^1	2.32	2.20	0.12
Average Noise = 2.18; σ = 0.09			
60 min. Incubation			
10^{7*}	3.49	2.56	0.93
10^5	2.45	1.84	0.61
10^4	2.69	2.08	0.61
10^3	2.40	2.01	0.39
10^2	2.31	1.96	0.35
10^1	2.20	2.15	0.05
Average Noise - 2.19; σ = 0.30			

* Represents a single determination

for the two incubation intervals (30 and 60 minutes). Figures 11 and 12 graphically depict the data. Table 22 is a listing of the original experimentation, from which the summary data were derived.

The sensitivity of the PIA test, upon initial examination, appears to have decreased from 10^{-1} EID_{50}/ml (based on the initial studies) to 10^{2} EID_{50}/ ml. Closer examination found that the apparent decrease in sensitivity was due to an increased efficiency in antigen production. The sensitivity criterion used to evaluate the PIA technique against known quantitation methods for the influenza virus is the egg infective dose, 50% endpoint (EID_{50}). This is a valid unit of comparison if the percentage of infective to noninfective virus remains the same from passage to passage. During the initial passages while adapting the virus to an egg-host system, small quantities of infectious virus were produced, but in the process, large quantities of noninfective antigen were also produced. These noninfective virus particles were not detected by the conventional EID_{50}, but being antigenic, they provided reactivity when measured with the PIA technique. As the number of passages increased, the virus became more adapted to the egg-host system which was evidenced by the increasing infectivity titers from lot to lot. The infective to noninfective virus ratio became larger while the total number of antigenic units remained relatively the same. The net effect of the increase of infective to noninfective viruses is a decrease in PIA sensitivity when related to an infective unit measurement. This phenomenon would also prevent the absolute correspondence of the specific signal for a given virus concentration when different agent preparations are used. This can be circumvented by utilizing the virus from a single pass when making other variable evaluations. To ensure that the sensitivity decrease was not a function of the "new antisera", an early pass (P3) was again examined. The sensitivity of the PIA was equivalent to the previously reported values (10^{-1}), substantiating that the apparent loss in sensitivity was a function of the antigen preparation.

Even though this occurred, a comparison can be made as to the effect of the incubation time on the test sensitivity. Examination of Figures 11 and 12 indicates that the sensitivity of the PIA technique increases with increasing incubation time. The sensitivity approximations for the incubation times are as follows: (1) 30 min. $\simeq$ 8×10^{2} EID_{50}/ml; and (2) 60 min. $\simeq$ 2×10^{2} EID_{50}/ml. With a thorough optimization of the

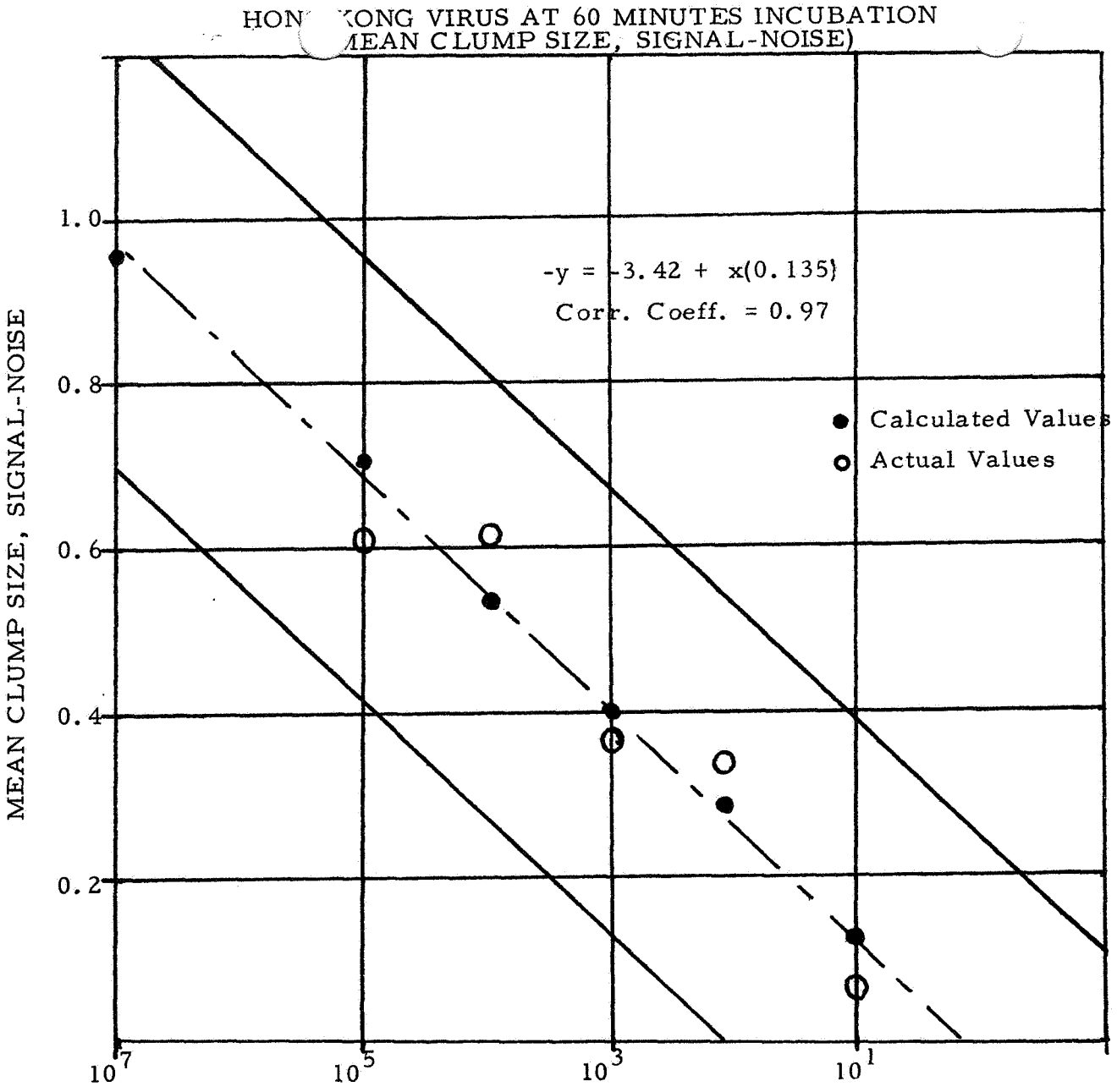


FIG. 11- MYXOVIRUS CONCENTRATION EID_{50}/ml

PIA SENSITIVITY DETERMINATION FOR INFLUENZA A_2
HONG KONG VIRUS AT 30 MINUTES INCUBATION
(MEAN CLUMP SIZE, SIGNAL-NOISE)

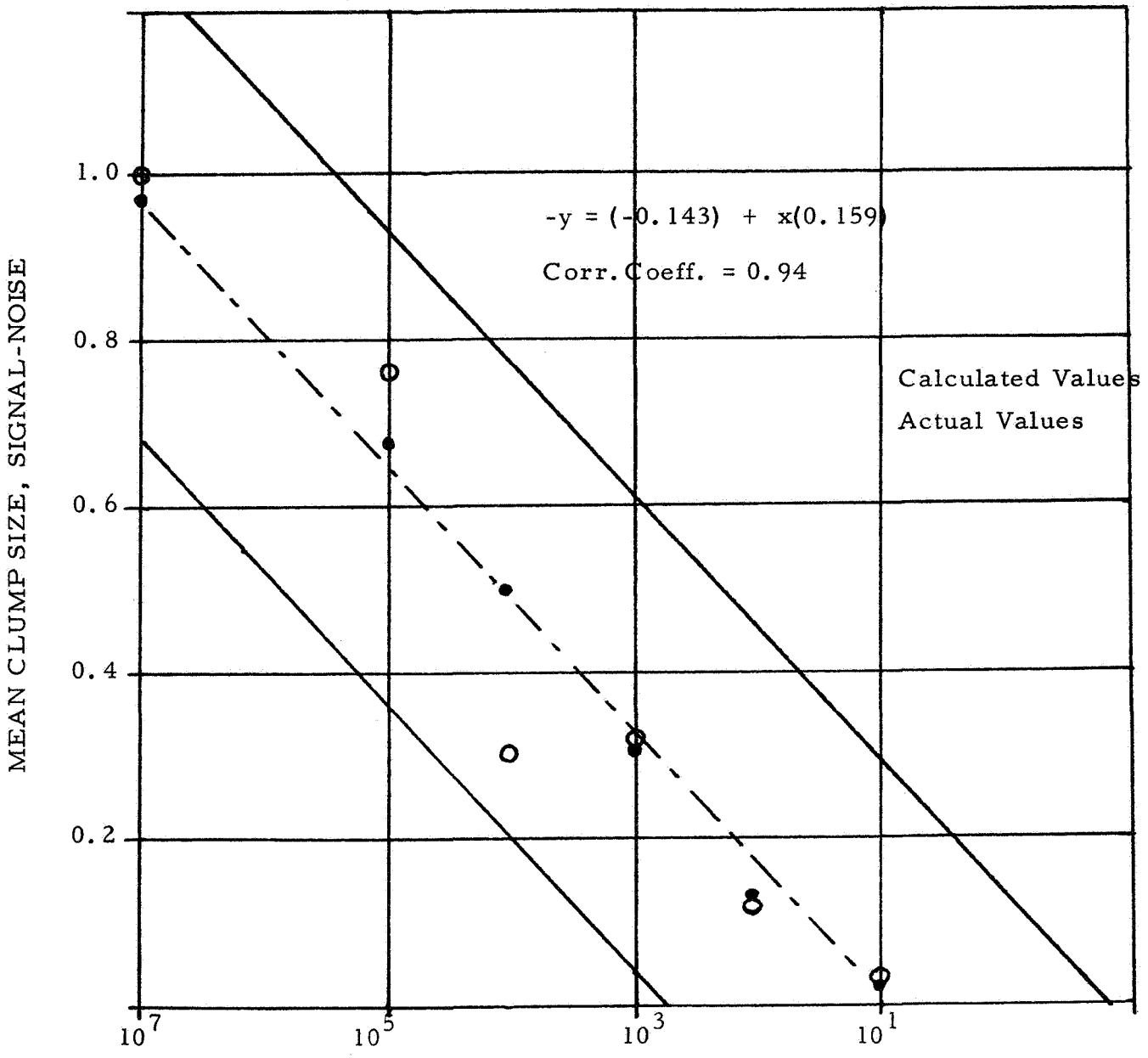


FIG. 12 - MYXOVIRUS CONCENTRATION EID_{50}/ml

Table 22

PIA SENSITIVITY DETERMINATIONS WITH THE MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration EID_{50}/ml)	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)
10^7	3.18	1.74	1.44	30
10^7	3.69	3.04	0.65	30
10^7	3.33	2.38	0.95	30
10^5	2.24	1.65	0.59	30
10^5	2.83	2.18	0.65	30
10^5	3.58	2.45	1.13	30
10^4	2.81	2.10	0.71	30
10^4	1.97	2.21	-0.24	30
10^4	2.33	1.96	0.37	30
10^4	2.57	2.31	0.26	30
10^4	2.99	2.87	0.12	30
10^4	2.49	2.50	-0.01	30
10^4	2.81	2.30	0.51	30
10^4	3.13	2.05	1.08	30
10^4	1.31	1.32	-0.01	30
10^4	1.98	1.57	0.41	30
10^3	1.97	1.61	0.31	30
10^3	2.22	2.17	0.05	30
10^3	2.48	2.29	0.19	30
10^3	2.40	2.01	0.39	30
10^3	2.26	2.28	-0.02	30
10^3	2.58	2.33	0.25	30
10^3	2.97	2.65	0.32	30
10^3	3.18	2.11	1.07	30
10^2	1.82	1.58	0.24	30
10^2	2.06	2.19	-0.13	30
10^2	1.84	2.27	-0.43	30
10^2	2.15	2.04	0.11	30
10^2	2.48	1.86	0.62	30
10^2	2.31	2.08	0.23	30
10^2	2.55	2.31	0.24	30
10^2	3.31	2.84	0.47	30
10^1	1.81	1.50	0.31	30
10^1	2.52	2.62	-0.10	30
10^1	1.98	2.09	-0.11	30
10^1	2.61	2.44	0.16	30
10^1	2.34	1.82	0.52	30
10^1	2.81	2.61	0.20	30
10^1	2.24	2.08	0.16	30
10^1	2.25	2.48	-0.23	30
10^0	2.08	1.99	0.09	30
10^0	2.04	2.38	-0.34	30

Table 22 (Continued)

PIA SENSITIVITY DETERMINATIONS WITH THE MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration (EID 50/ml)	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)
10^7	3.49	2.56	0.93	60
10^5	3.04	1.85	1.19	60
10^5	2.44	1.72	0.72	60
10^5	2.87	1.72	1.15	60
10^5	2.20	1.57	0.63	60
10^5	1.77	2.24	-0.47	60
10^4	2.40	1.96	0.44	60
10^4	2.69	2.37	0.32	60
10^4	4.23	2.64	1.59	60
10^4	2.81	2.44	0.37	60
10^4	3.00	2.26	0.71	60
10^4	2.04	1.43	0.61	60
10^4	1.42	1.28	0.14	60
10^4	2.52	1.89	0.63	60
10^4	2.70	1.68	1.02	60
10^4	2.41	1.93	0.48	60
10^4	2.68	1.96	0.72	60
10^4	2.31	2.17	0.14	60
10^4	2.20	1.82	0.38	60
10^4	4.27	2.64	1.63	60
10^3	2.44	2.64	-0.20	60
10^3	1.93	1.72	0.21	60
10^3	2.12	1.73	0.39	60
10^3	2.47	1.69	0.67	60
10^3	0.55	1.60	-0.05	60
10^3	2.18	1.66	0.52	60
10^3	2.35	2.18	0.17	60
10^3	3.08	2.37	0.71	60
10^3	2.04	2.61	-0.56	60
10^3	2.26	1.92	0.34	60
10^3	2.97	2.53	0.44	60
10^3	3.27	2.73	0.54	60
10^3	2.35	1.49	0.86	60
10^3	1.29	1.27	0.02	60
10^3	2.01	1.92	0.09	60
10^3	2.32	1.87	0.45	60
10^3	2.36	1.98	0.38	60
10^3	2.26	1.66	0.60	60
10^3	1.58	1.32	0.26	60
10^3	3.85	3.51	0.34	60
10^2	3.73	2.50	1.23	60
10^2	2.09	1.72	0.37	60
10^2	1.67	1.72	-0.05	60
10^2	1.88	1.57	0.31	60
10^2	2.08	1.78	0.30	60
10^2	2.19	1.64	0.45	60
10^2	2.15	1.69	0.46	60

Table 22 (Continued)

PIA SENSITIVITY DETERMINATIONS WITH THE MYXOVIRUS AS DETERMINED BY MEAN CLUMP SIZE

Myxovirus Concentration (EID 50/ml)	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)
10^2	2.34	1.91	0.43	60
10^2	2.74	2.29	0.45	60
10^2	1.69	1.91	-0.22	60
10^2	2.68	2.29	0.39	60
10^2	2.55	1.88	0.67	60
10^2	2.63	2.20	0.43	60
10^2	2.09	1.97	0.12	60
10^2	2.23	1.60	0.63	60
10^2	2.34	1.94	0.40	60
10^2	2.12	1.90	0.22	60
10^2	3.84	3.48	0.36	60
10^2	2.44	2.23	0.21	60
10^1	1.76	1.78	-0.02	60
10^1	1.65	1.83	-0.18	60
10^1	2.44	1.73	0.71	60
10^1	1.47	1.51	-0.04	60
10^1	1.82	1.99	-0.17	60
10^1	1.88	1.69	0.19	60
10^1	1.94	1.88	0.06	60
10^1	2.55	1.89	0.66	60
10^1	2.60	2.33	0.27	60
10^1	2.75	1.98	0.77	60
10^1	2.37	2.53	-0.16	60
10^1	1.98	1.95	0.03	60
10^1	1.98	2.03	-0.05	60
10^1	1.85	1.97	-0.12	60
10^1	3.46	4.78	-0.32	60
10^0	2.77	2.47	0.30	60
10^0	2.04	2.42	-0.38	60
10^0	2.18	2.61	-0.43	60
10^0	1.82	1.93	-0.11	60
10^0	2.48	2.74	-0.26	60
10^0	2.69	3.11	-0.42	60

reaction condition variables, the sensitivity should approach the desired range of 10^0 - 10^1 EID_{50}/ml (even in a preparation with a high ratio of infective to noninfective virus).

Propagation Effort and Antigen Purification

Efforts were continued to characterize the Myxovirus (influenza A_2 Hong Kong strain). A ten-fold volume concentration (with Diaflo ultrafiltration apparatus) on preparations P5, P5-1A and P5-1B was completed. The results are listed in Table 23.

Table 23

Effect of Volume Concentration on HA Activity and Egg Infectivity

	Quantity (ml)		EID_{50}/ml		HA	
Passage	Before	After	Before	After	Before	After
P_5	260	25	3.2×10^7	3.2×10^8	1:64	1:256
P_{5-1A}	670	65	3×10^7	3.2×10^8	1:128	1:024
P_{5-1B}	700		not done	not done	1:128	1:1024

The 700 ml harvest of P_{5-1B} was divided into aliquots of 500 and 200 ml. Both parts were concentrated tenfold by Diaflo ultrafiltration. The residual of the 500 ml aliquot was adsorbed with barium sulfate-sodium oxalate and eluted in gelatin fortified phosphate buffer. This eluate was divided into two parts. One was centrifuged against a 3-60% sucrose gradient at 37,000 rpm for 90 minutes (SW #39 swinging bucket rotor, Model L2 Spinco ultracentrifuge). The other part was spun against a cushion consisting of three layers: 1 ml each of 60, 40 and 30% sucrose. The same conditions and time of spin were used for this aliquot as for the first.

The 200 ml aliquot of the original material was Diaflo ultrafiltered through three changes of phosphate buffer, pH 7.2. The HA deteriorated from 1:512 for the first concentration to 1:256 after the third change. The protein of the original harvest of P_{5-1B} was 2 mg/ml. After barium sulfate-oxalate adsorption and elution, the protein content was 0.75 mg/ml. The eluate fluid (containing gelatin, which gave a positive Lowrey test) had a protein content of 0.5 mg/ml. The barium sulfate adsorption technique removes most of the protein from allantoic fluid concentrated by Diaflo ultrafiltration.

A typical profile of the density gradient and cushion centrifugation are listed in Table 24.

Table 24

Myxovirus Purification by Density Gradient and Cushion Centrifugation

Density Gradient 3% - 60% Sucrose			Cushion 30, 40 and 60% Sucrose (1 ml each)		
Increasing Density	Fraction	HA	Increasing Density	Fraction	HA
↓	1	1:0	↓	1	1:0
	2	1:2		2	1:0
	3	1:512		3	1:2048
	4	1:512		4	1:8192
	5	1:64		5	1:1024
	6	1:16		6	1:1024
	7	1:16		7	1:1024
	8	1:8		8	1:128
	9	1:32		9	1:32
	10	1:32		10	1:0
				11	1:0
				12	1:256

In all cases, the top most portion of each tube of the sucrose revealed some HA activity. It is most probable that this HA activity is due to the presence of incomplete virus and/or viral protein. Protein determinations were not conducted on the fractions from the gradients because the size of each sample was too small.

4.2.2 MYCOPLASMA PNEUMONIAE

Sensitivity Studies

The purpose of these studies, as with Myxovirus, was to examine the effect of high-quality antibody and varying incubation times on the sensitivity and reliability of the PIA test. The characterization of the antibody used to sensitize the bead preparation is described in Section 4.1.

The summarized results of the sensitivity determinations at the three incubation times are described in Table 25. Table 26 is a listing of the individual experiments, from which the summary data were derived. The sensitivity of 100 CFU/ml is at least one order of magnitude lower than achieved during the initial studies with this organism. The increases in sensitivity is quite dramatic, and can be primarily attributed to the improved quality of the antiserum.

The Mycoplasma antigen used for this test series was frozen and thawed several times before use. This procedure also contributed to the increased sensitivity. The physical action of the freeze-thaw cycle serves to break up the cell wall of the organism into smaller but more numerous fragments. This effectively increases the amount of reactive particulates. To further increase the test sensitivity, more vigorous sample fragmentation may be required. The scatter in the data indicates that the fragmentation process (under these conditions) was not consistent in the quantity of particulates formed per organism. Reliability can be increased by closely controlling the conditions which are utilized for cell disruption.

Conclusions cannot be drawn regarding the effect of incubation time on the test sensitivity. Linearity (signal vs. concentration) must be reestablished before this type of comparison can be performed. The one log increase in sensitivity demonstrates the successful preparation of high-quality antibody. It is anticipated that special methods in antigen pretreatment will provide the reliability and sensitivity required to utilize the PIA test as an environmental monitor for Mycoplasma pneumoniae.

Antigen Production and Purification

A total of 11 liters of M. pneumoniae were produced since the last reporting period. The entire volume was concentrated 50 fold and assayed at an average titer of 5×10^8 CFU/ml. The resultant 220 ml was further reduced in volume 10 fold. No assay was conducted on the final concentrate.

The M. pneumoniae was routinely monitored for purity by growth inhibition with M. pneumoniae antiserum impregnated filter paper discs, growth on PPLO agar containing methylene blue and reduction of 2, 3, 5-triphenyltetrazolium chloride.

Table 25

SUMMARY, PIA SENSITIVITY DETERMINATIONS WITH THE MYCOPLASMA AS DETERMINED BY MEAN CLUMP SIZE

Concentration (CFU/ml)	Signal (S)	Noise (N)	S-N
30 min. Incubation			
10^{5}*	2.19	1.68	0.51
10^{3}	4.16	3.76	0.40
10^{2}	4.50	3.66	0.84
10^{1}	3.72	3.61	0.11
60 min. Incubation			
10^{7}	1.86	1.63	0.23
10^{5}	3.65	2.72	0.93
10^{3}	3.30	2.76	0.54
10^{2}	2.75	2.61	0.14
10^{1}	3.64	2.81	0.83
90-120 min. Incubation			
10^{7}*	2.41	1.91	0.50
10^{5}*	2.27	1.82	0.45
10^{3}	3.17	2.02	1.15
10^{2}*	2.65	1.89	0.76
10^{1}	2.38	1.87	0.51

* Represents a single determination

Table 26

PIA SENSITIVITY DETERMINATION WITH THE MYCOPLASMA AS DETERMINED BY MEAN CLUMP SIZE

Mycoplasma Concentration (CFU/ml)	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)
10^5_3	2.19	1.68	0.51	30
10^3_3	4.21	3.08	1.13	30
10^3_2	4.16	4.45	-0.29	30
10^2_2	4.24	3.58	0.66	30
10^2_1	4.77	3.64	1.13	30
10^1_1	4.10	3.56	0.54	30
10^1	3.34	3.67	-0.33	30
10^7_7	2.34	1.80	0.54	60
10^7_7	1.72	1.56	0.16	60
10^7_5	1.52	1.14	-0.02	60
10^5_5	4.05	2.77	1.28	60
10^5_5	2.17	1.79	0.38	60
10^5_5	5.34	3.10	2.24	60
10^5_5	1.62	1.40	0.22	60
10^5_5	1.72	1.40	0.32	60
10^3_3	7.00	5.86	1.14	60
10^3_3	6.52	5.77	0.75	60
10^3_3	4.59	3.38	1.21	60
10^3_3	2.08	1.74	0.34	60
10^3_3	2.46	2.53	-0.07	60
10^3_3	3.09	2.54	0.55	60
10^3_3	2.35	1.67	0.68	60
10^3_3	2.29	1.77	0.52	60
10^3_3	1.52	1.64	-0.12	60
10^3_3	1.62	1.65	-0.03	60
10^2_2	6.52	4.90	1.60	60
10^2_2	2.45	2.52	-0.07	60
10^2_2	4.72	4.32	0.40	60
10^2_2	1.77	1.84	-0.07	60
10^1_1	2.09	1.77	0.32	60
10^1_1	5.29	3.58	1.71	60
10^1_1	3.96	3.22	0.74	60
10^1_1	1.94	1.67	0.27	60
10^1_1	5.48	4.51	0.97	60
10^1_1	6.22	4.62	1.60	60
10^1_1	2.87	2.61	0.26	60
10^1_1	4.51	3.06	1.45	60
10^1_1	2.14	1.82	0.32	60
10^1_1	2.23	1.58	0.65	60
10^1	1.79	1.44	0.35	60

Table 26 (Continued)

PIA SENSITIVITY DETERMINATION WITH THE MYCOPLASMA AS DETERMINED BY MEAN CLUMP SIZE

Mycoplasma Concentration (CFU/ml)	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)
10^7	2.41	1.91	0.50	90-120
10^5	2.27	1.82	0.45	90-120
10^3	2.56	1.70	0.86	90-120
10^3	3.78	2.44	1.34	90-120
10^2	2.65	1.89	0.76	90-120
10^1	2.42	1.89	0.53	90-120
10^1	2.35	1.76	0.59	90-120

4.3 SPECIFICITY OF THE IMPROVED ANTISERA

The specificity of the improved antisera for both the myxovirus and the Mycoplasma was tested with three heterologous organisms. Table 27 is a summary of the results obtained from the cross reactivity tests. Table 28 is a compilation of the individual experiments in the specificity studies. The results indicate that there was no significant cross reaction between the improved antisera and the heterologous agents. However, the limit of specificity cannot be determined since closely related organisms were not tested. Certain amounts of cross reactivity would be anticipated when antigenically similar organisms are tested with a single antiserum.

4.4 CLINICAL SAMPLE TESTING

Four contractually required clinical samples were received from NASA in addition to five additional samples that were to be tested if possible. Each sample was assayed by the PIA undiluted, or at a 1:10 dilution. The summary results are shown in Table 29. (Actual experimental data are in Table 30). The first four samples (labeled A, B, C, and D) were positive, while samples E-H were negative. Sample J demonstrated an intermediate positive result. The PIA results do not correspond to results obtained by the conventional methods. In the conventional procedure, aliquots of the unknowns were aseptically injected in the amniotic sac of 11-day old embryonated hens eggs. After 72 hours, the amniotic fluids were harvested and tested for the presence of virus by screening for hemagglutination activity. Samples A and D demonstrated HA activity, while samples B and C were negative. Hemagglutination inhibition titers for the positive samples were not obtained. These results do not correspond completely with the PIA data; however, variations in the constituents of the sample suspending fluid most probably account for this lack of correlation. It is felt that correlation between the two methods is a simple attainable goal, to be achieved through the development of a suspending fluid which is most compatible with the PIA test.

Table 27

CROSS REACTIVITY DETERMINATIONS USING AVERAGE SIGNAL MINUS NOISE OF MEAN CLUMP SIZE

Agent Concentration		Sensitized Beads: Mycoplasma	Sensitized Beads: Myxovirus
Mycoplasma	10^4 CFU/ml	+1.00	-0.19
Myxovirus	10^4 EID_{50}/ml	-0.06	+0.68
Adenovirus	10^4 $TCID_{50}$/ml	-0.31	-0.13
Herpesvirus	10^4 PFU/ml	-0.23	-0.06

Table 28

CROSS REACTIVITY DATA SUMMARY
INCUBATION 60 MINUTES AT 40°C

Antibody Sensitized Beads	Antigen for Cross Reaction		Mean Clump Size Signal	Noise	S-N
Mycoplasma	Mycoplasma 10^4 CFU/ml		3.63	2.22	1.41
			3.08	2.50	0.58
		Ave.			+1.00
	Myxovirus 10^4 EID_{50}/ml		2.41	2.54	-0.13
			2.56	2.58	-0.02
			1.97	1.99	-0.02
		Ave.			-0.06
	Adenovirus 10^4 $TCID_{50}$/ml		2.58	2.85	-0.27
			2.56	2.76	-0.20
			1.82	2.27	-0.45
		Ave.			-0.31
	Herpesvirus 10^4 PFU/ml		2.58	2.89	-0.31
			2.91	3.28	-0.37
			2.16	2.18	-0.02
		Ave.			-0.23
Myxovirus	Myxovirus 10^4 EID_{50}/ml		2.81	2.26	0.55
			2.62	1.75	0.87
			2.59	1.97	0.62
		Ave.			+0.68
	Mycoplasma 10^4 CFU/ml		2.15	2.60	-0.45
			1.85	2.17	-0.32
			2.87	2.69	0.18
		Ave.			-0.19
	Adenovirus 10^4 $TCID_{50}$/ml		2.66	2.37	0.29
			2.08	2.27	-0.19
			2.02	2.52	-0.50
		Ave.			-0.13
	Herpesvirus 10^4 PFU/ml		2.32	2.76	-0.44
			2.27	2.34	-0.07
			2.15	1.98	0.17
			2.03	1.92	0.11
		Ave.			-0.06

Table 29

SUMMARY, PIA EXAMINATION OF CLINICAL SAMPLES*

Unknown Designation	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N
A	2.54	1.36	1.18
B	2.38	1.35	1.02
C	2.40	1.26	1.14
D	2.64	1.26	1.38
E	1.18	1.24	-0.06
F	1.28	1.29	-0.01
G	1.24	1.25	-0.01
H	1.34	1.25	0.09
J	1.83	1.25	0.58

* Average S-N at 60 minutes incubation, Sample undiluted.

Table 30

PIA EXAMINATION OF CLINICAL SAMPLES

Unknown Designation	Signal Mean Clump Size (S)	Noise Mean Clump Size (N)	S-N	Incubation Time (minutes)	Dilution for Test
A	2.46	1.32	1.14	60	Undiluted
A	2.62	1.40	1.22	60	Undiluted
A	1.37	1.46	-0.09	60	1:10
A	1.30	1.44	-0.14	30	Undiluted
A	1.48	1.59	-0.11	180	1:10
B	2.19	1.30	0.89	60	Undiluted
B	2.56	1.40	1.16	60	Undiluted
B	1.74	1.51	0.23	60	1:10
B	1.55	1.58	-0.03	180	1:10
C	2.26	1.13	1.13	60	Undiluted
C	2.53	1.38	1.15	60	Undiluted
C	1.42	1.33	0.09	60	1:10
C	1.52	1.60	-0.08	180	1:10
D	2.64	1.26	1.38	60	Undiluted
D	2.30	1.47	0.83	60	1:10
D	1.69	1.49	0.20	60	1:10
D	1.55	1.46	0.09	180	1:10
E	1.18	1.24	-0.06	60	Undiluted
E	1.29	1.28	0.01	180	1:10
F	1.28	1.29	-0.01	60	Undiluted
F	1.34	1.28	0.06	60	1:10
G	1.24	1.25	-0.01	60	Undiluted
G	1.26	1.25	0.01	180	1:10
H	1.34	1.25	0.09	50	Undiluted
H	1.33	1.33	0.00	180	1:10
J	1.97	1.16	0.81	60	Undiluted
J	1.69	1.33	0.36	60	Undiluted

4.5 EXAMINATION OF READOUT TECHNIQUES FOR THE PIA TEST

4.5.1 MANUAL READOUT SYSTEMS

Terminal Dilution Methods

The most common method of quantitating the latex agglutination reaction involves the observation of an all-or-none reaction (agglutination) using sequential dilutions of either antigen or antibody to provide an end-point. The technique has been extensively used for measurement of antibody titers. The highest dilution of antibody that reacts with antigen-coated particles to form agglutinates is identified as the titer. In practice, reaction sensitivities and specificities have been found to be at least equal to other more conventional serological tests. The latex method has an inherent advantage in this regard. Since one of the reactive species (usually antigen) is adsorbed on a particle, its size is effectively that of the carrier. Thus, fewer effective antigen-antibody combinations are needed to produce a visible or otherwise measurable reaction than in conventional precipitation or agglutination methods.

Microscopic Method

Microscopic readout of the PIA test was used throughout the MEMS program. This readout involved counting the number of clumps in each microscopic field and determining the number of beads per clump. A particle size distribution analysis was then made on the results of these data. This analysis resulted in a readout in which the measurement of all the clumps was reduced to a single value termed the mean clump size. The derivation of mean clump size is shown in Appendix B.

A disadvantage of the microscopic particle size distribution method is the time required for performing and reading the agglutination reaction. Instrumentation of the readout phase is seen as the last logical step toward producing a method of great practical utility for detecting agent antigen in clinical samples.

4.5.2 SEMI-AUTOMATED READOUT SYSTEMS

Electronic Particle Counters

Electronic sensing zone instruments have been widely used in recent years for quantitation of particle numbers and size distribution of microbial populations, red and white blood cells, etc.

In this study, it was found that volume distribution measurements made by the Coulter Counter were seriously distorted, the frequency of large volumes being greatly exaggerated. The causes of these distortions were not attributed to any inherent nonlinearity in the signal amplification, or to a physical particle coincidence count in the sensing zone, but to a combination of electronic coincidence errors in the amplifier and a more serious effect of wide distributions of transit times through the sensing zone.

A Celloscope was used in our laboratory to determine the feasibility of automating the readout by counting the clumps using the conductometric method. The Celloscope is a particle counter that works on the Coulter Counter principle (see diagram in Figure 13). A tube with a 48 μ hole is placed in a solution containing the particles. The tube is filled with a conductive solution, an electrode is placed inside the tube and in the sample solution. Current then travels from the solution outside to the solution inside the tube through a 48 μ orifice. If no particles pass through the orifice, a steady state current is maintained through the hole. However, if a particle of sufficient size passes through the orifice, the current flow is restricted for the duration of the passage. This restriction of current flow, which is proportional to particle size, is then measured as an impulse and recorded through suitable electronics on a counter and printer.

Liquid passes from the outside to the inside of the tube by applying a vacuum to the tube. The flow rate of the sample solution through the orifice can be regulated by regulating the vacuum. The amount of current and the subsequent amplification can be regulated by the appropriate controls present in the machine.

Preliminary runs were made with the Celloscope in which sensitized beads were mixed with the appropriate antigen and the resulting mixtures counted on the particle counter.

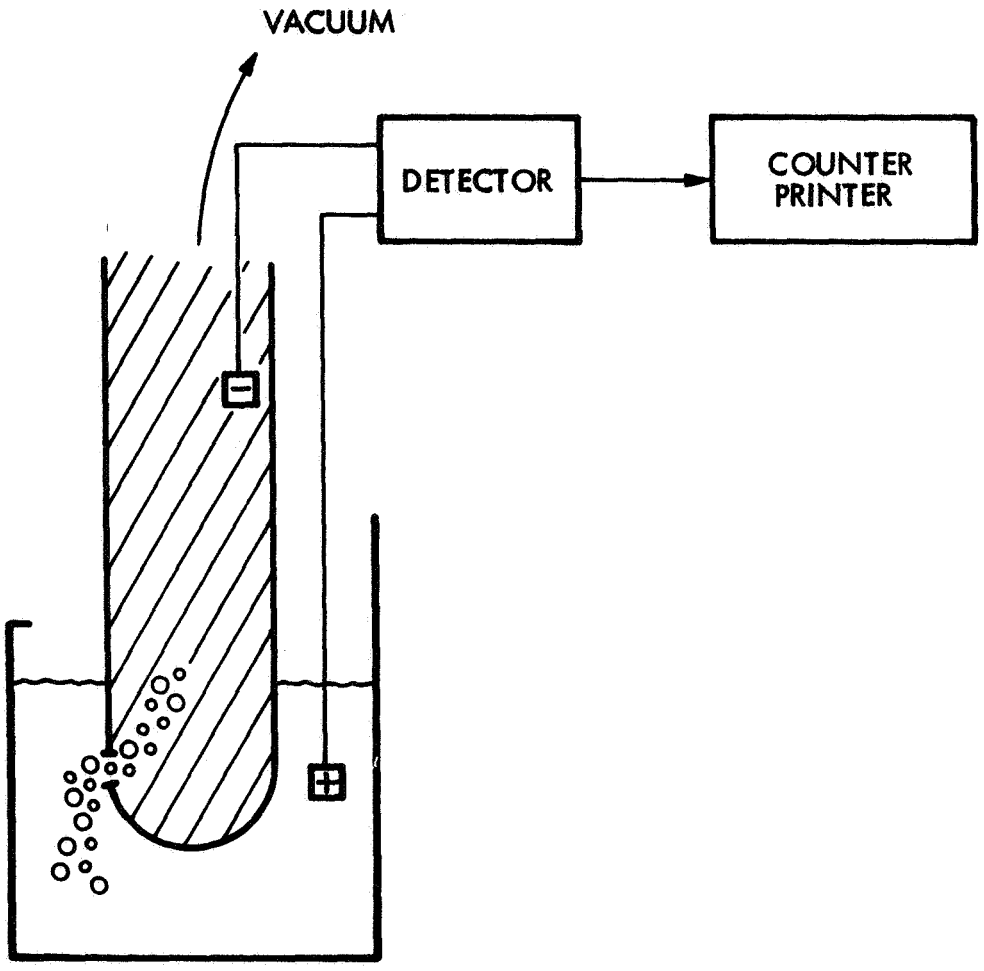


Figure 13 - Schematic of Coulter Principle

Figure 14 represents a preliminary determination of the feasibility of the Celloscope to monitor the PIA reaction. The control had a mean clump size of 1.45 while the sample was 4.01, as measured with the PIA (manual readout). The initial data indicate that the Celloscope can be utilized in this application, but the lower sensitivity limits must be determined. Further examination will have to be performed on the variables involved in conductomatic counting before a final decision is made.

Light Scattering Particle Counters

A second method examined was a light scattering device developed by Royco. This device (Figure 15) operates by measuring the change in light intensity across a 200 micron tube which contains a solution of particles moving in a single direction. The amount of change in light intensity is directly proportional to the size of the particle or particles that pass through the tube. The data presented in Figure 16 show the difference between the control and sample(same preparation analyzed by the Celloscope). The curves represent the distribution of the particle sizes in the different channels of the analyzer. At the clump sizes that were present in the control and sample, the Royco was dramatically able to differentiate between the two. The simplicity of operation and the rapidity of the data presentation were two inherent advantages with this device. It remains to be seen on further testing whether smaller differences between control and sample can be detected.

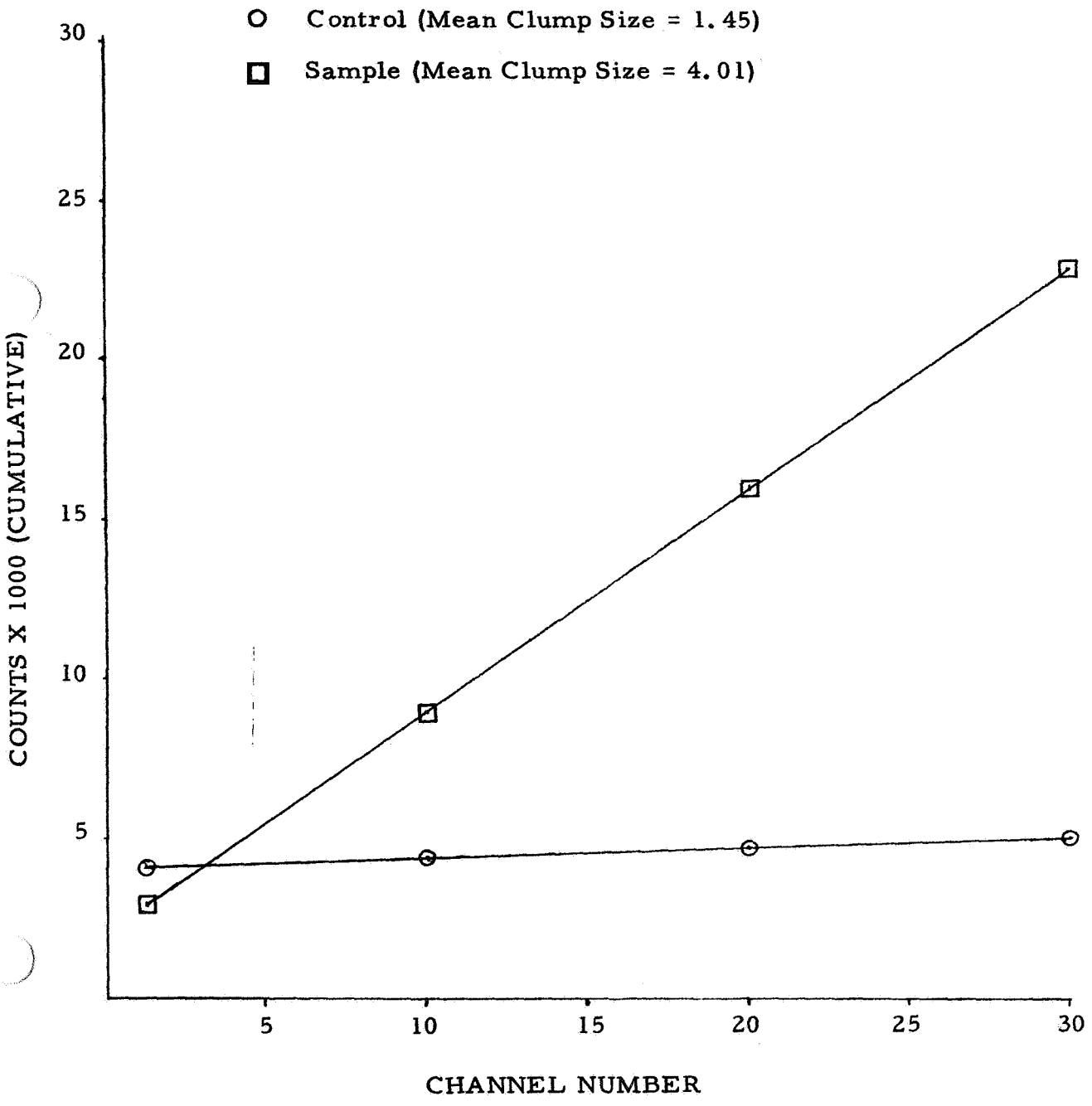


FIGURE 14, CELLOSCOPE PRELIMINARY EVALUATION DATA

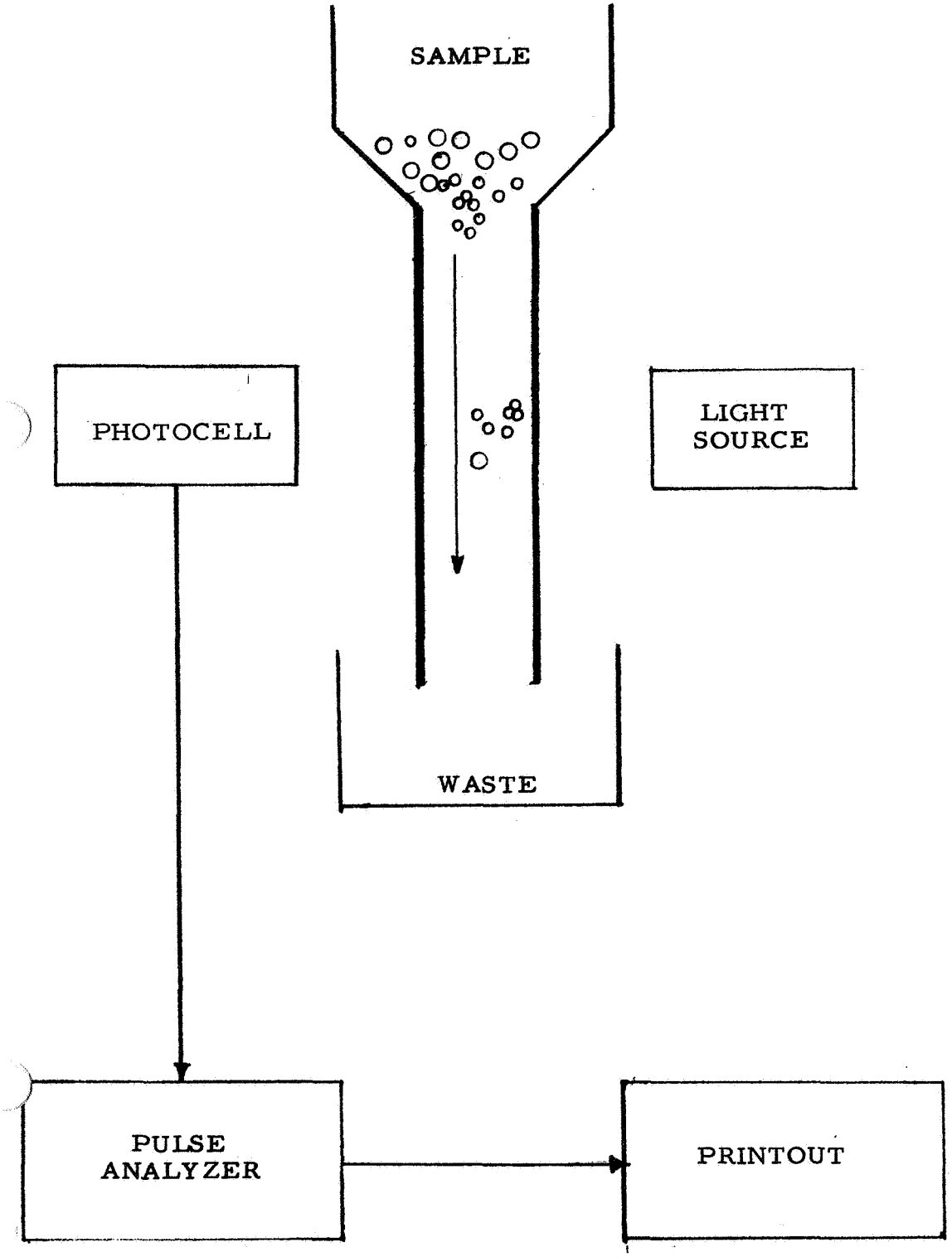


FIGURE 15 - SCHEMATIC - ROYCO LIGHT SCATTERING SYSTEM

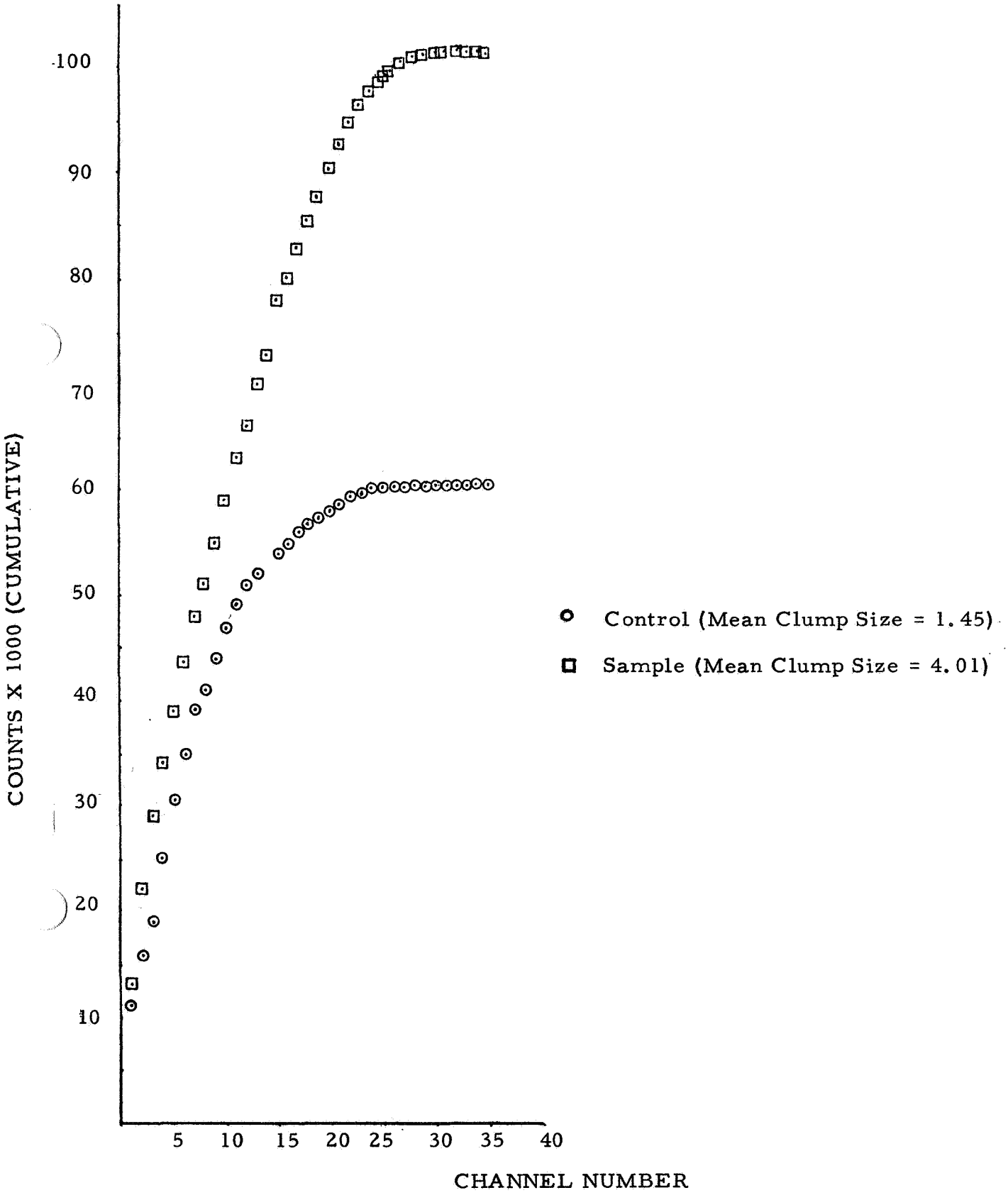


FIGURE 16 - ROYCO PRELIMINARY EVALUATION DATA

Automated Microscopic Method

The system shown schematically in Figure 17 demonstrates the third method of counting that was examined. This system utilizes a microscope for the initial magnification. A sample is placed on the stage of the microscope and its image is detected by the video camera. This signal is then processed by computer modules and displayed on the TV screen monitor. The sample may also be viewed directly through the eyepiece of the microscope.

Selected parameters of the particles in the sample are measured by computer modules and presented in alphanumeric form at the top of the monitor screen. Detailed measurements can be made on individual particles as well as entier fields.

The counting of particles is controlled by a logic that follows the rules for manual counting, leading to statistically valid results. The advantage of this system is that rapid operation requires only minimum technical and manipulative skills. The current disadvantage with this system is the need for a microscope that has to be focused for each sample. Maintaining the microscope in focus and field selection are performed manually.

Table 31 presents the desirable properties of a counting system for the PIA test and rates each counting system against these properties. The selection of the optimum automated readout system must await further evaluation.

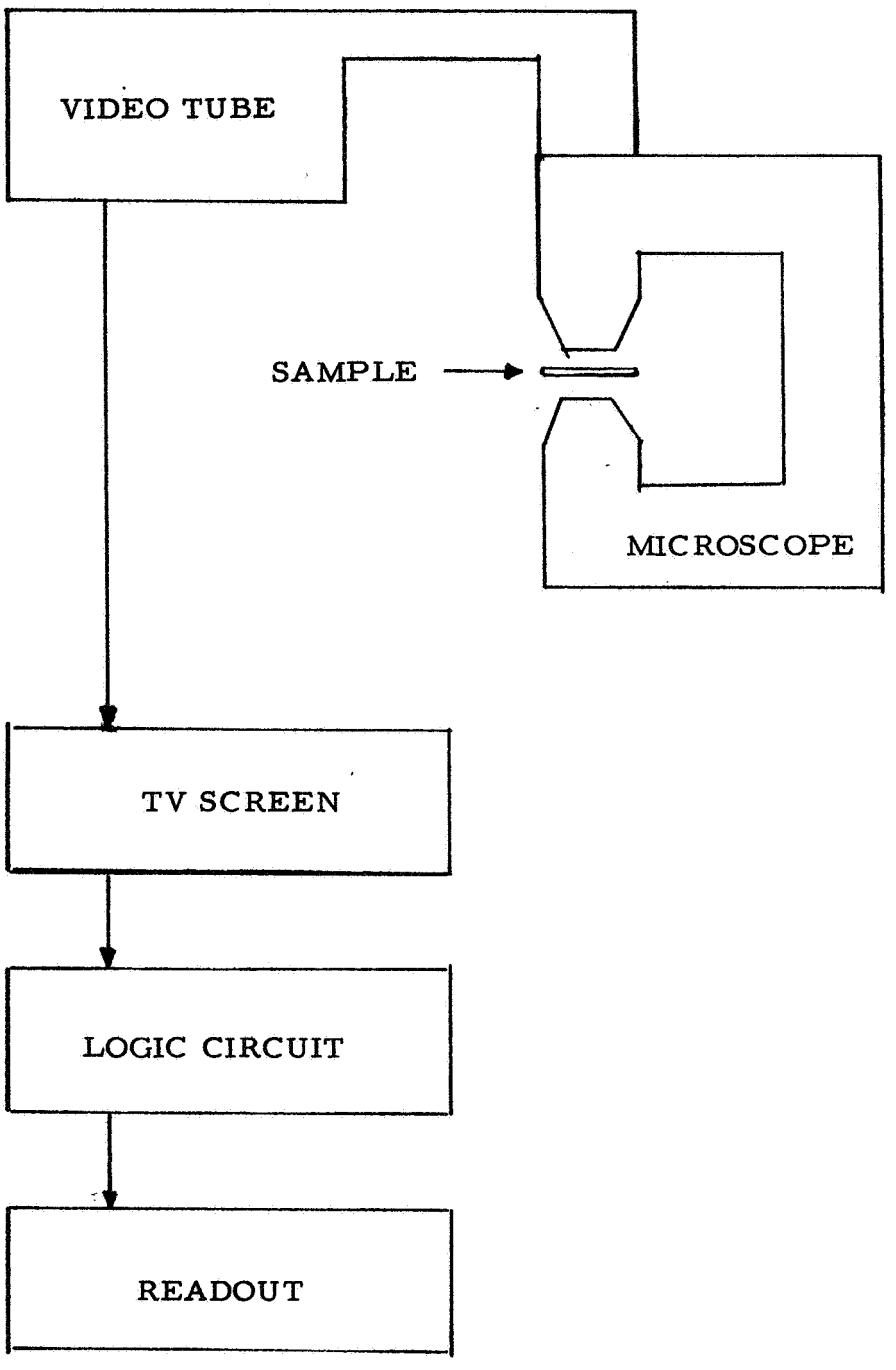


FIGURE 17 - SCHEMATIC OF AUTOMATED MICROSCOPE READOUT

Table 31

PRELIMINARY EVALUATION - AUTOMATED READOUT DEVICES

Readout Method	Response Time	Ease of Operation	Sensitivity*	Particle Size Range
Terminal Dilution	4-18 hours	fair	10^2 - 10^4 EID_{50}/ml	>0.2 μ
PIA	15 minutes	fair	1-10 EID_{50}/ml	>1 μ
Electronic Sensing Zone (Celloscope)	2-5 minutes	good	10-100 EID_{50}/ml**	>1 μ
Light Scattering (Royco)	2-5 minutes	excellent	10-100 EID_{50}/ml**	>2 μ
Automated Microscope (Millipore)	1 minute	good	10-100 EID_{50}/ml**	>0.5 μ

* Infectivity of Influenza A_2 Hong Kong virus expressed as egg infective doses - 50% endpoint per ml (EID_{50}/ml)

** These values represent the results of a limited number of experiments, where lower sensitivities were not evaluated. A sensitivity and reliability comparison among these three methods or in relation to the manual PIA method cannot be accomplished with these data. These results do indicate that the methods are applicable and merit extensive analysis.

5.0 CONCLUSIONS AND RECOMMENDATIONS

The PIA test has been evaluated for 5 viruses and 1 Mycoplasma species. The sensitivity of the test for the 5 viruses is high enough to make the test useful for ecological monitoring. The sensitivity of the PIA test for Mycoplasma has not as yet reached its maximum, but indications are that the sensitivity can be raised to a level necessary to permit ecological monitoring.

The PIA test has been shown to be a feasible method to rapidly detect and identify small quantities of virus in aqueous suspensions. It has also been shown that the sensitivity of the PIA test is dependent on a number of factors. Some of these factors are: 1) antigen size, 2) antibody titer, and 3) suspending fluid.

Conventional methods for identification of viruses involve such methods as the HA, HAI and CF techniques. These methods required relatively large amounts of viral antigen for their successful operation.

Conventional methods of isolation involve the use of living hosts. In such cases, the material being isolated and detected is an infective unit. The PIA test, on the other hand, does not detect infective units as such, but rather antigenic units. These antigenic units may be of much smaller size than the infective units (i.e., viral structural components), In some cases, these units may be two or three orders of magnitude more numerous in a virus preparation than the infective units themselves. This is due to the inefficiency of the cellular propagation process itself or to the results of environmental inactivating stresses. It is this amplification that provides the unique sensitivity of the PIA test.

With larger antigens such as the Mycoplasma, the detection of the antigenic unit versus the detection of the infective entity can work to a disadvantage. The multiplication of Mycoplasma apparently leaves fewer defective antigens in solution than occur in virus multiplication. As a result of fewer free antigenic particulates, it becomes necessary to break up the larger agent into smaller pieces, each of which will serve equally well as antigen for the PIA test.

The amount of antibody that can be placed on a latex particle represents an important element in the overall sensitivity of the PIA system. The amount of specific antibody on a sensitized bead can be

increased in two ways: 1) by an increase in the amount of gamma globulin on the bead, and 2) by an increase in the purity of the antibody. The greatest potential improvement is in increasing antibody purity. Examinations in our laboratory have shown that both the reliability and the sensitivity of the test can be increased by a proportionate increase in antibody titer. The sensitivity of the PIA test for Mycoplasma pneumoniae increased one order of magnitude by increasing the CF titer of the antibody by a factor of approximately 20.

The suspending fluid of the virus is also important in determining the overall sensitivity of the PIA test. One of the factors that affect detection sensitivity is the background or noise level. The nonspecific agglutination of the sensitized particles is due to a net attractive electrostatic charge comprised of the surface charge (due to protein) plus the charge on the polystyrene bead itself. By suitably varying the pH and ionic strength of the reaction mixture, one can decrease nonspecific agglutination while maximizing specific clumping.

In summary, the overall sensitivity of the PIA test for the viruses has been shown to surpass or equal the results obtained with conventional methods. Specificity studies have indicated no significant cross reactivity among the agents tested. The feasibility of the PIA test for clinical specimen determinations has been established. Three systems were evaluated for automating the readout, all of which demonstrated compatibility with the PIA method.

The following tasks are recommended so that the value of the PIA test (high sensitivity and short response time) can be applied, not only as an ecological viral monitor, but as a method able to perform real time diagnostic tests.

1. Continue reagent improvements to increase test sensitivity and specificity.
2. Optimize the process variables to increase test sensitivity and reliability.
3. Select and optimize a suitable readout system.
4. Develop a breadboard device, incorporating the readout system with sample processing hardware.
5. Expand the utility of the PIA test to multiagent analysis, bacterial detection and antibody quantitation.

REFERENCES

1. Singer, J.M. and Plotz, C.M. The Latex Fixation Test. I. Application to the Serologic Diagnosis of Rheumatoid Arthritis. Am. J. Med. 21, 888-892 (1956).

APPENDIX A

ANTIBODY CHARACTERIZATION AND PURIFICATION

HEMAGGLUTINATION (HA) USING THE MICROTITER METHOD*

1. Place 50 µl of standard PBS in each of 12 wells of a V-bottomed Microtiter plate.

2. Add 50 µl of stock virus to the first well and mix.

3. Using 50 µl diluters, serially dilute the first 11 wells.

4. To each tube, add 50 µl of a 0.5 per cent erythrocyte suspension (in PBS).

5. Cover the Microtiter plate, shake gently and incubate 20-45 min. at 37°C.

6. Examine the bottom of the wells for agglutination and record as follows:

   + bottom of well covered by a layer of finely clumped cells of granular appearance; complete agglutination.

   ± a slight ring of partially agglutinated cells.

   0 A small, sharply outlined button of cells in the center of the tube; no agglutination.

7. The virus HA titer is the reciprocal of the highest dilution of virus causing + (complete) agglutination.

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*E.H. Lennette, 1964. General Principles in Diagnostic Procedures for Viral and Rickettsial Diseases (3rd Ed.). Am. Pub. Health Assoc: New York, p. 57

HEMAGGLUTINATION INHIBITION (HAI) USING THE MICROTITER METHOD

1. Inactivate all sera at 56°C for 30 minutes.

2. Add 25 µl of PBS to each well, except the first well.

3. Add 25 µl of test serum diluted 1:8 to the 1st, 2nd and last well (serum controls).

4. Using 25 µl diluters, begin with the 2nd well and serially dilute the contents of each well except the last.

5. Add 25 µl of an antigen dilution containing 4 HA units to each well except the serum control.

6. Shake gently and allow to stand at room temperature for 30 minutes.

7. Add 25 µl of 0.5 per cent erythrocytes in PBS (chicken, sheep or human) to each well. Shake gently and allow to stand for 1 hour at room temperature.

8. Read for agglutination patterns, as for the HA procedure. The serum HAI titer is the reciprocal of the highest dilution yielding complete negative (0) agglutination.

HA - HAI TEST REAGENTS

1. Phosphate buffered saline (PBS) pH 7.2, with 0.01 M phosphate.

2. Erythrocyte suspension - 0.5% washed chicken, sheep or human red blood cells in PBS.

3. Antigen - 4 hemagglutinating (HA) units in 25 µl of PBS. One HA unit is the highest twofold dilution of antigen which completely agglutinates a 0.5% suspension of red blood cells.

COMPLEMENT FIXATION TEST

A. Hemolysin Titration

1. Add 50 µl of the appropriate hemolysin dilution to each well in the series.

2. Add 50 µl of guinea pig complement diluted 1:30 to each well.

3. Add 50 µl diluent to each well.

4. Add 50 µl of 2 percent sheep cells to each well.

5. Incubate 1 hour at 37°C.

6. Read for cell lysis.

B. Complement Titration

1. Add 50 µl of the appropriate complement dilution to each well in the series.

2. Add 50 µl of diluent to each well.

3. Add 25 µl of antigen (2 units) to each well.

4. Incubate 1 hour at 37°C.

5. Combine equal volumes of hemolysin (2 units) and 2% sheep cells, let react for 15 minutes. Add 50 µl to each well.

6. Incubate 1 hour at 37°C.

7. Read for cell lysis.

HEMOLYSIN TITRATION

Well No.	Hemolysin (μl)	(dilution)	Complement 1:30 (μl)	Diluent (μl)	Sheep Cells 2% (μl)	Incubate
1	50	1:1000	50	50	50	1 hr at 37°C
2		1:2000				
3		1:3000				
4		1:4000				
5		1:5000				
6		1:6000				
7		1:8000				
8		1:10000				
9		1:12000				
10		1:16000				

COMPLEMENT TITRATION

Well No.	Complement (μl)	(dilution)	Diluent (μl)	Ag. 2 units (μl)	Incubate	*SRBC	Incubate
1	25	1:10	50	25	1 hr. at 37°C	50	1 hr. at 37°C
2		1:15					
3		1:20					
4		1:25					
5		1:30					
6		1:35					
7		1:40					
8		1:50					
9		1:60					
10		1:70					

*Sensitized sheep red blood cells; i.e.,
equal volumes of hemolysin (2 units) and 2% sheep RBC's
combined and reacted for 15 minutes @ 37°C.

C. Diagnostic Complement Titration Test

1. The test antiserum is serially diluted* (with appropriate two-fold dilutions) using the Microtiter 25 µl Microdiluters; make six such dilutions.

2. To each serial dilution antiserum well is added 25 µl of the test antigen (2 units) and 50 µl of complement (2 units).

3. Wells 7, 8, and 9 contain the first three antiserum dilutions without the added antigen.

4. Well 10 contains antigen without antiserum.

5. Wells 11 and 12 contain no antigen and no antiserum.

6. Mix gently and incubate for 18 hours at 6-8°C. Remove from refrigerator and incubate at 37°C for 15 minutes.

7. Mix equal amounts (by volumes) of 2 units of hemolysin and a 2% suspension of freshly-prepared sheep red blood cells. React for 15 minutes at room temperature, then add 50 µl to each of the first eleven wells. To the 12th well add 25 µl of sheep cells without hemolysin.

8. Mix and incubate for 30 minutes at 37°C.

9. Read for cell lysis.

*All diluent is composed of Veronal buffer containing 0.02% gelatin.

DIAGNOSTIC COMPLEMENT TITRATION TEST

Well No.	Antiserum (μl)	(dilution)	Antigen, 2 units (μl)	Complement 2 units (μl)	Diluent (μl)	Incubate	Sensitized RBC's 2 units hemolysin + 2% sheep cells (μl)
1	25	1:4	25	50	0	18 hrs. at 6°-8°C	50
2	25	1:8	25	50	0		50
3	25	1:16	25	50	0	Then 15 min. at 37°C.	50
4	25	1:32	25	50	0		50
5	25	1:64	25	50	0		50
6	25	1:128	25	50	0		50
7	25	1:4	0	50	25		50
8	25	1:8	0	50	25		50
9	25	1:16	0	50	25		50
10	0	-	0	50	25		50
11	0	-	25	50	50		50
12	0	-	0	50	75		24 (w/o hemolysin)

All reagents are made or diluted in Veronal buffer containing .02% gelatin. A standard Microtiter is used with disposable titer plates (u-plates) and disposable Micropipettes.

STEP 1

GLOBULIN ISOLATION
VIA 1/2 AMMONIUM SULFATE PRECIPITATION

1. Dilute serum in an equal amount of saturated $(NH_4)_2SO_4$. The saturated salt is added drop by drop, stirring lightly while adding at 4^oC.

2. Store overnight at 4^oC.

3. After 21-24 hours centrifuge the mixture at 10,000 rpm for 15 minutes.

4. Decant the supernate (but save for further precipitation, if needed).

5. Resuspend precipitate in a minimal amount of saline.

6. Dialyze against phosphate buffered saline, pH 8.2, for 24 hours. *

* Alternate Method:

Dialyze for 2 hours against running tap water, then dialyze against glycine buffer overnight.

STEP 2

GLOBULIN ISOLATION VIA (1/3 AMMONIUM SULFATE PRECIPITATION)

1. With constant stirring, slowly add (dropwise) to a 50 ml serum sample a total of 25 ml saturated ammonium sulfate solution, thus effecting one-third saturation. During the first stages, do not proceed with the addition of the salt solution until all precipitate from the previous addition has dissolved. Eventually the precipitate persists. Continue adding the salt solution slowly. (When large samples of antiserum are to be fractionated, a pH of 7.8 should be maintained at all times during the precipitation procedure. Constant maintenance of the pH is not necessary with amounts below 50 ml.)

2. Upon completion of the addition of the ammonium sulfate solution, adjust the pH of the suspension to 7.8 with 2 N NaOH. Small samples are adjusted with weak alkali (0.3 to 1.0 N) and large samples, 100 ml or more, with stronger alkali (4.0 to 5.0 N). The use of strong alkali on small samples may result in local excess of alkali with subsequent denaturation of the protein, while the use of weak alkali on large samples may dilute the system to the point where gamma globulin will begin to dissolve.

3. Continue stirring the suspension for an additional 2 hours in order to avoid mechanical trapping of serum components other than gamma globulin in the precipitate; let stand overnight $4^{\circ}C$.

4. Centrifuge the suspension for 30 min. at 1400 xg. (about 3000 rpm with a rotating radius of 14 cm).

5. Dissolve the precipitate in enough saline to restore the volume of the original serum sample.

6. Remove the ammonium sulfate from the precipitate by dialyzing against PBS, pH 8.2, for several days at $4^{\circ}C$. Change the dialysate twice daily checking for ammonium ions, by the addition of a few drops of Nessler's reagent.

7. After dialysis is complete remove the solution from the tubing. Centrifuge the solution at $4^{\circ}C$ for 30 min. at 1400 xg. Some insoluble material usually forms during dialysis. The final solution will be slightly opalascent.

8. Check the product for yield of antibody protein, using any conventional method.

APPENDIX B

PIA TEST PROCEDURE
AND
BEAD AGGLUTINATION ANALYSIS

PASSIVE IMMUNO AGGLUTINATION (PIA) PROTOCOL

1. Mix .05 ml of 3×10^9/ml sensitized polystyrene latex beads (1.1 μ). diameter) with .05 ml of the appropriate antigen suspension.
2. Mix by shaking.
3. Incubate in a water bath for 60 minutes at $40^{\circ}C$.
4. Place an aliquot of the mixture on a slide and cover with a cover slip.
5. Inspect six randomly selected fields under a microscope and record clump size distribution in each field.
6. Take clump size distribution of six fields and perform bead agglutination analysis.

BEAD AGGLUTINATION ANALYSIS

The mean number of beads in the clumps is a pertinent measure of bead agglutination. A method of determining this parameter is presented below.

A dominant characteristic of bead agglutination is the mean clump size, or group size. In practice, it is usually not practical to take all the data required to exactly determine the mean clump size, so that an approximation method is required.

Define a few pertinent parameters:

G	The number of particles in a given clump
N	The total number of clumps, all sizes
η_j	The observed number of clumps of size G = j
p(G)	The probable number of clumps of size G
P(>G)	The probable number of clumps larger than G
M(>G)	The total number of particles in clumps larger than G
ΔN(>G)	The number of clumps larger than G

A few relations follow immediately:

$$N = \sum_{j=1}^{\infty} \eta_j \tag{1a}$$

$$P(>G) = P(>G-1) + p(G) \tag{2a}$$

$$P(>0) = 1.0 \tag{2b}$$

$$\bar{G} = \frac{\sum_{j=1}^{\infty} G_j \, p(G_j)}{\sum_{j=1}^{\infty} p(G_j)} \tag{3}$$

Also, the probabilities p(G) are normalized such that

$$\sum_{j=1}^{\infty} p(G_j) \equiv N \tag{1b}$$

Thus the total number of particles (beads) in all clumps is

$$M = N\bar{G} = \sum_{j=1}^{\infty} G_j \, p(G_j) = \sum_{j=1}^{\infty} j \; \eta_j \tag{4}$$

(Note that $G_j \equiv j$.)

The parameter of most basic interest is the mean clump size, $\bar{G}$. In principle, $\bar{G}$ may be experimentally determined from Equation 3. However, in practice it is difficult or impossible to accurately determine the sizes of the very large clumps (e.g., $G > 10$). Therefore it will be necessary to choose a statistical model to approximately describe the distribution of large clumps.

Suppose, for example, the clump sizes were randomly (Poisson) distributed, having mean clump size $\bar{G} = \bar{m}$. Then

$$\frac{1}{N} p(G) = \frac{\bar{m}^G e^{-\bar{m}}}{G!} = \frac{\bar{G}^G e^{-\bar{G}}}{G!} \tag{5a}$$

The percent of clumps of size greater than G will be

$$\frac{P(>G)}{N} = \frac{1}{N} \sum_{j=G+1}^{\infty} p(G_j) = 1 - \frac{1}{N} \sum_{j=0}^{G} p(G_j)$$

$$= 1 - \sum_{j=0}^{G} \frac{\bar{m}^j e^{-\bar{m}}}{j!} \tag{5b}$$

Or, counting beads, the percent of beads in clumps of size greater than G will be

$$\frac{M(>G)}{M} = \frac{1}{N\bar{G}} \sum_{j=G+1}^{\infty} G_j\, p(G_j) = 1 - \frac{1}{\bar{m}} \sum_{j=0}^{G} j\, \frac{\bar{m}^j e^{-\bar{m}}}{j!} \qquad (5c)$$

Equations 5b and 5c are shown in Figures 1 and 2 for selected values of the mean clump size $\bar{G} = \bar{m}$.

If the clumps were Poisson distributed in size Figure 1 illustrates that counting the number of clumps >G might be an unsatisfactory criterion. If the mean size were as small as 3.0 the number of clumps greater than, say 8 beads, is expected to be less than 1%. Yet for a mean size of 16.0 - just over five times as large - the probability is over 97%. The sensitivity in this region would be greatly enhanced. But the scale would be non-linear, and the dynamic range would be limited.

A different probability distribution has been tentatively selected, described by

$$P(>G) = N\left(1 - \frac{G}{G_a}\right), \quad G \leq G_a \qquad (6a)$$

This implies

$$p(G) = \frac{N}{G_a}, \quad G \leq G_a \qquad (6b)$$

For this distribution the mean clump size, $\bar{G}$, is approximately

$$\bar{G} = \frac{\sum_{j=1}^{G_a} j\, \frac{N}{G_a}}{\sum_{j=1}^{G_a} \frac{N}{G_a}} = \frac{\frac{N}{G_a}\left[\left(G_a + \frac{1}{2}\right)^2 - \left(\frac{1}{2}\right)^2\right]}{N} \simeq \frac{G_a}{2} \qquad (6c)$$

The total number of particles in clumps greater than size G is expected to be

$$M(>G) = \sum_{j=G+1}^{\infty} G_j\, p(G_j)$$

$$= M - \sum_{j=1}^{G} G_j\, p(G_j) = M - \sum_{j=1}^{G} j\, \frac{N}{G_a}$$

$$= N\bar{G} - \frac{N}{G_a}\left[\frac{\left(G+\frac{1}{2}\right)^2 - \left(\frac{1}{2}\right)^2}{2}\right] \simeq \frac{N G_a}{2} - \frac{N G^2}{2 G_a} \qquad (6d)$$

Assume an experiment in which the number of clumps, η_j, in each size group has been accurately counted up to same size $G = \hat{G}$, and the total number of clumps ΔN, greater than size $\hat{G}$ have also been counted. If from this data the parameter G_a can be estimated, then (using Equation 6d) the mean clump size $\bar{G}$ can be approximately determined.

If in Equation 6a the experimentally determined $\Delta N(>G)$ is substituted for $P(>G)$, then it can be solved for G_a in terms of the known parameters $\hat{G}$, N, and ΔN:

$$G_a \simeq \frac{\hat{G}}{1 - \frac{\Delta N}{N}} \qquad (7)$$

Finally, $\bar{G}$ is calculated from Equations 3 and 6D

$$\bar{G} = \frac{M}{N} = \frac{\sum_{j=1}^{G} G_j\, \eta_j}{N} + \frac{M(>G)}{N}$$

$$\simeq \frac{\sum_{j=1}^{\hat{G}} j\, \eta_j}{N} + \frac{G_a}{2}\left(1 - \frac{\hat{G}^2}{G_a^2}\right) \qquad (8)$$

where G_a is determined from Equation 7.

Current evaluation of the data is performed on an XDS 940 computer through a time sharing linkup. The program for processing the data is written in standard Fortran IV language. This program takes the number and size of clumps counted in each of the six fields and determines the mean clump size. This program not only performs the necessary calculations but prints out the appropriate tables and corresponding graphs resulting in a considerable savings in time shown (as examples) in Tables 1 and 2 and Figure 1. The calculations performed by the program to determine the mean clump size are derived from the general formula shown in 1 and 2. This formula represents a refinement of the steps used in the program on the Olivetti 101.

1.) G_a = X axis Intercept

$$G_a = \left(\frac{1}{2} + \frac{\hat{G}}{P}\right) + \sqrt{\left(\frac{1}{2} + \frac{\hat{6}}{P}\right)^2 - \frac{\hat{G}\,(\hat{G}+1)}{P}}$$

where $p \equiv \underline{p}$ $\left(> \hat{6} \tilde{-} 1 - \frac{\triangle N\,(\geq \hat{6})}{N}\right)$

2.) $\overline{G}$ = corrected mean clump size

$$\overline{G} = \sum_{j=1}^{\hat{G}} jn_j + \frac{1}{G_a - 1}\left[\frac{G_a^2}{3} - \frac{1}{12} - \frac{1}{12 G_a} + 2\left(\frac{\hat{G}+1}{2}\right)^3 - \overline{\left(G + \frac{1}{2}\right)^2}\right]$$